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Effects of Nitrogen Sources in Phytase Production on Bacterial Strains Isolated from Malaysia's Hot Spring

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ABSTRACT

Efficient strategies for phytase production gained increasing importance as more applications require high amounts of phytase for the market. Four phytase-producing bacterial strains isolated from Malaysia's hot springs were used in this study to determine the effect of nitrogen sources on phytase production. All of the strains were screened out by applying halozone method which shows all of the strains were definitely positive phytase producer. Phytase Screening Medium (PSM) with soybean extract as substrate was used as a cultivation medium. Optimised condition with 1.0 % (w/v) of glucose (as carbon source), pH 5.5 and 37°C temperature was applied. Yeast extract and peptone were used to identify optimum nitrogen source in maximum phytase production. Quantitative analysis observed were optical density, colony forming unit, pH values and phytase activity to identify the effect of nitrogen source in phytase production. The finding was bacterial strain L3 as the best producer in producing maximum phytase (0.2162 U/mL) with optimised condition using yeast extract as nitrogen source. Findings in this study proved that yeast extract act as the optimum nitrogen source which contribute to maximum phytase production as supported by previous studies. This study can provide an efficient strategy to produce maximum phytase as few studies stated that phytase is an application tool in functional food production that consists of myo-inositol phosphates that is believed to have important pharmacological effects.

Keywords: Phytase, Phytic acid, Nitrogen source, Peptone, Yeast extract

INTRODUCTION

Major storage form of phosphorus and inositol in plant seeds is phytate which is an anhydrous salt form of phytic acid. Phytases can be found in several organisms such as microorganisms, plants, fungi, yeast and some in animal tissues. Microorganism of phytase producer can be found in rhizospheric soil of crop such as *Aspergillus ficuum*, *Escherichia coli* and *Bacillus subtilis* (Alias et al., 2018). Phosphomonoesterase such as phytase is capable in hydrolysing phytic acid to inorganic orthophosphate (Pi) and a series of lower esters of myo-inositol thereby,

producing free myo-inositol (Ramachandran et al., 2005). Hydrolysis of phytic acid catalysed by phytases into mono-, di-, tri-, tetra- and pentaphosphates of myo-inositol and inorganic phosphate (Mohamad et al., 2012).

Phytase involved in food production as myo-inositol phosphates have important pharmacological effects such as the impediment of diabetes complications, anti-inflammatory activity and anti-tumour effects. Excess unutilized inorganic phosphorous and phytases in animal manure and liquid effluent degraded by microorganisms lead to eutrophication of fresh water bodies. Generally, phytic acid chelates nutritionally important divalent cations (calcium, zinc and iron) and some proteins that rendering them biologically unavailable to the animals (Kumar et al., 2012). Microbial phytases being widely used as feed additives for swine and poultry dietary in purpose to improve utilization of plant-based feed and used in plants to increase the phosphate uptake. Improving nutritive quality of food and feed which contain phytic acid is the significant role of phytase (Alias et al., 2018). In term of human health, phytate-rich diets also give benefits to human such as prevention of kidney stone formation, protection from diseases like coronary heart disease, atheriosclerosis and cancers (Greiner & Konietzny, 2006). Application of D- myo-inositol (1, 2, 6) triphosphate in studies of diabetes complications prevention, chronic inflammations and cardiovascular diseases treatment being used. While application of myo-inositol(1,3,4,5,6) pentakisphosphate being suggested in anticancer therapeutic strategy due to its antiangiogenic and antitumour effects (Greiner & Konietzny, 2006). In biotechnological applications, phytase had increased of interest in feed manufacturing industry as an environment-friendly feed additive. The ability of phytase in binding to proteins and chelating minerals clearly shows the function of phytase as anti-nutrient. Enhanced protein digestibility and mineral availability can improve the nutritional value of plant-based foods by adding the phytase through phytate hydrolysis during digestion in the stomach or during food processing (Shobirin et al., 2010).

Generally, nitrogen source is one of an important element in production of phytase. This is due to the effect of the absence of nitrogen source that will greatly affect the growth of cells and phytase production (Banerjee, 2002). Cultivation medium which combination of 1% of yeast extract, 10% of soybean meal extract, 0.1% Tween 80 and citric acid shows high optical density of cells (OD_{600} = 6.0) and phytase activity (213.1 U/gm) (Dharmsthiti et al., 2005). Next, production of maximum phytase was obtained by using 1% of yeast extract followed by other nitrogen source such as potassium nitrate. Peptone and yeast extract are widely used in phytase production involving *Aerobacter aerogenes* and *Klebsiella aerogenes* (Sasirekha, 2012). According to Singh et al. (2013), a better phytase production (398.0 U/mL) was obtained by using yeast extract as nitrogen source. Yeast extract, peptone and tryptone are said to show stimulated maximum production of phytase (Soni & Khire, 2007). A mixture of yeast extract and wheat bran stimulate production of phytase and growth of *Pseudomonas fluorescens* which proved by maximum phytase activity. While in *Aspergillus niger*, maximum phytase being produced when using peptone (Tungala et al., 2013). Furthermore, maximum phytase (0.264 U/mL) was produced when using yeast extract followed by asparagine (0.248 U/mL) and peptone (0.21 U/mL) (Bajaj & Wani, 2011).

The significance of this study is to provide a strategy to produce maximum phytase by optimising the nitrogen sources as phytase had many beneficial effects on organisms such as human, animals and plants. The main purpose of this research was to find the best nitrogen source for the production of phytase from different potential phytase-producing bacterial strains.

MATERIALS AND METHODS

Bacterial Sample

In this study, four phytase producing bacterial were previously isolated by Alias et al. (2018) from different locations of hot springs in Malaysia which were Ulu Legong, Kedah (strain A), Ranau, Sabah (strain B9), Labis, Johor (strain L3) and Dusun Tua, Selangor (strain RT). Yeast extract and peptone being used to identify the effects of nitrogen sources in phytase production on each of the four isolated potential phytase bacterial. Other

optimum condition used in this study were glucose as carbon source (Sasirekha, 2012), pH 5.5 (Selvamohan et al., 2012) and temperature 37°C (Alias et al., 2018).

Production of Phytase Enzyme

Luria Bertani (LB) media was used to grow the isolates overnight and 1 mL of inoculum from this primary culture was added to a conical flask consist of 100 mL of Phytase Screening Medium (0.4 % soybean extract, 1.0 % (w/v) glucose, 1% nitrogen source (yeast extract and peptone),0.05 % (w/v) potassium chloride (KCl), 0.05 % (w/v) magnesium sulfate ($MgSO_4.7H_2O$), 0.5 % ammonium nitrate (NH_4NO_3), 0.01 % manganese sulphate monohydrate ($MnSO_4.H_2O$), 0.5 % (w/v) calcium chloride ($CaCl_2.2H_2O$), 0.01 % iron sulfate ($FeSO_4.H_2O$), 0.1 % citric acid and 0.1 % Tween 80 as described by Dharmsthiti et al. (2005). At 37°C with 180 rpm in incubator shaker, the culture was incubated at different time intervals (24, 48 and 72 h) to determine the amount of phytase produced. Phytase assay being carried out to determine the amount of phytase production. Samples were centrifuged for 20 min at 4°C to harvest enzyme produced by taking the clear cell-free supernatants which was then being centrifuged at 10,000 x g for phytase assay.

Optimisation of Nitrogen Source

Growth analysis

1 mL of samples being taken at different time intervals (every hour of the first 8 h, 24, 48 and 72 h). Optical density (OD) was used to determine the growth of bacterial cells by measuring its turbidity at 600 nm wavelength. Colony Forming Unit (CFU) also was used to determine the cultivability of the bacterial cells at different time intervals (1, 5 and 24 h). 100 µL of culture was serially diluted in 900 µL of phosphate buffered saline (PBS) from 10^4 to 10^7 and plated on Nutrient Agar plates which then being incubated at 30°C for 24 hours.

pH values

pH reading was observed at different time intervals (0, 24 and 72 h) to determine the changes in pH values of the culture. pH 5.5 was the initial pH of the culture.

Phytase assay

To measure the released amount of phosphate from sodium phytate during the enzymatic reaction, ammonium molybdate method as described by Rocky-Salimi et al. (2016) being applied in this study to determine the phytase activity. Firstly, a reaction mixture consists of 400 µL of 1.5 mM sodium phytate in 100 mM Tris- HCl buffer (pH 7.0) and 100 µL of crude enzyme was incubated at 55°C for 30 min. Then, 400 µL of color reagent solution consist of (1.5:1.5:1 ratio of 0.24 % ammonium vanadate, 10 % ammonium molybdate, 65 % nitric acid) was added and centrifuged again at 15,000 × g for 10 min at room temperature to stop the reaction. The mixture developed into yellow colour due to the phytase activity and was measured spectrophotometrically at 415 nm wavelength. A standard curve prepared from potassium dihydrogen phosphate KH_2PO_4 was used to determine the phytase activity produced (Rocky-Salimi et al., 2016).

Statistical Analysis and Measurement

This study consists of two different treatments of nitrogen sources (peptone and yeast extract) treated on four different bacterial strains. All experimental conditions in this study were made in duplicates. Microsoft Excel Software 2019 (Microsoft 365) was used to analyse the statistical data 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 to determine the *P* value and group means were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Growth Analysis

Two of the quantitative analysis being measured in this study were optical density (OD) and colony forming unit (CFU). Twelve points of sampling being taken at different time interval (every hour of the first 8 hours, 24, 48 and 72 hours) during the fermentation to obtain the growth curve for all the bacterial strains. All these bacterial strains (strain RT, L3, B9 and A) were isolated at different locations that require growth curve to understand their life cycle. CFU was measured using colony counter meter. Three points of sampling being taken at different time interval (1, 5 and 24 hours) during fermentation.

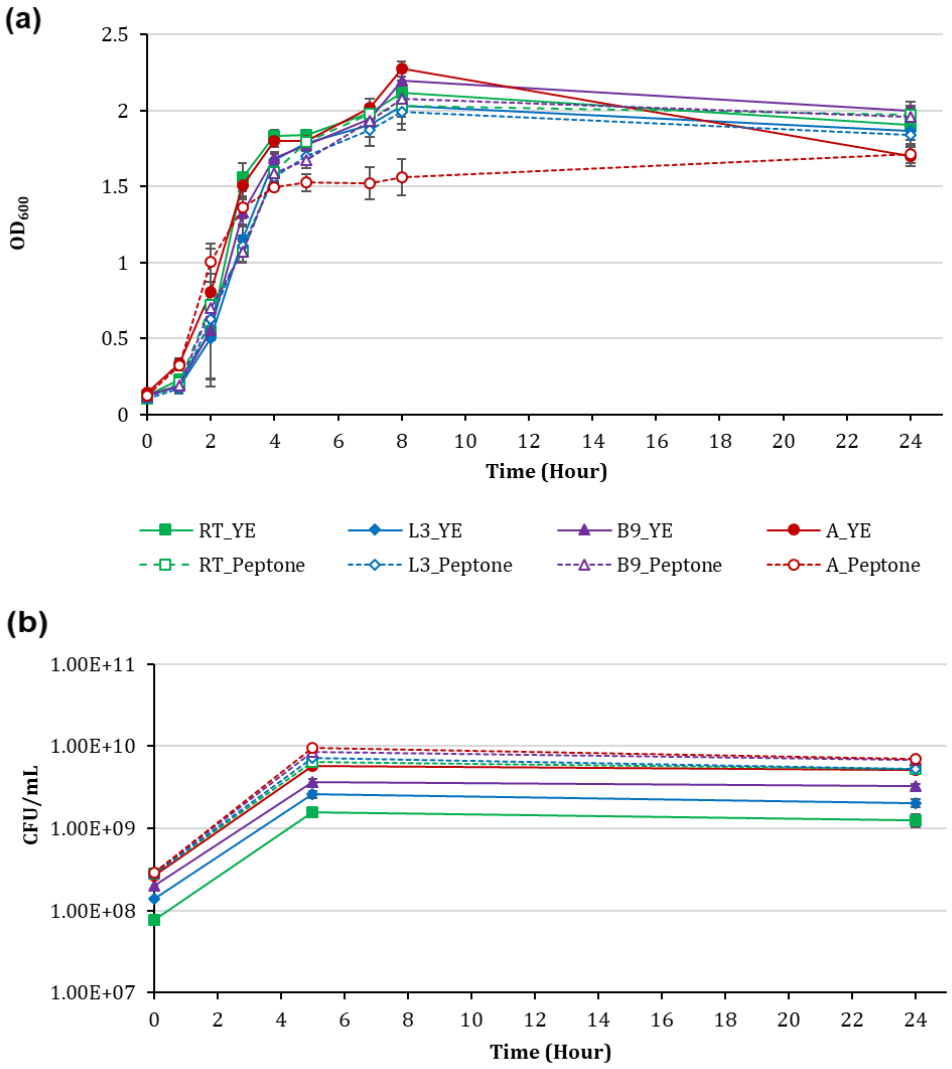

Fig. 1. Correlation of all strains growth for the study of nitrogen source optimisation for production of phytase, evaluated by (a) OD_{600} measurement, and (b) $CFU.mL^{-1}$ counting. All data shown are mean values from replica flasks with error bars are ±1 of standard deviation.

Fig. 1(a) shows the effect of yeast extract and peptone on phytase bacterial growth incubated at 37°C for 24 hours. Generally, all strains in peptone fermentation were in lag phase during the early first hour of fermentation followed by exponential phase (1 h to 8 h). Next, during 8 h to 24 h, all bacterial cells were in stationary phase

except for strain A with peptone which was in stationary phase since 4 h of fermentation. An assumption had been made that strain A did not favour peptone as nitrogen sources that being used in this study. Growth pattern of bacterial cells in yeast extract fermentation is quite similar to the growth of cells in peptone fermentation. Between both yeast extract and peptone fermentation, all strains showed no significant difference in their OD reading with *P* value of L3 (P=0.468), RT (P=0.423), B9 (P= 0.441) and A (P=0.275). All strains were in declination phase after 24 hours. It can be observed that in Fig. 1 (a), strain A fermented in the medium using yeast extract as nitrogen source showed the highest OD compared to the other three strains. However, strain B9 had the highest OD in fermentation using peptone as nitrogen source.

As shown in Fig. 1, strain A in yeast extract fermentation shows the best growth compared to other strains, while peptone fermentation shows a contrast result where strain A has the lowest growth compared to other bacterial strains. 0.1% yeast extract along with 10% soybean meal extract, 0.1% Tween 80 and citric acid showed high optical density of bacterial cells (OD_{600} = 6.0), where complex medium formulated based on previous described PSM medium using soybean meal extract contributed to highest production of phytase to replace the phytic acid which is expensive (Dharmsthiti et al., 2005). 1% yeast extract resulting in maximum phytase production, thus in *Aerobacter aerogenes* and *Klebsiella aerogenes*, organic nitrogen source such as yeast extract and peptone are being extensively used (Sasirekha, 2012). Fig. 1(b) shows the effect of yeast extract and peptone on colony forming unit (CFU). In both yeast extract and peptone fermentation, strain A shows the highest viable counted colony formed followed by strain B9, L3 and RT. Higher amount of viable counted formed colony was observed on all bacterial strains fermented in peptone fermentation compared to bacterial strains fermented in PSM consist of yeast extract.

pH values

Measurement of pH values was one of the quantitative analysis being carried out to determine the changes in pH values of the culture. The degradation of phytic acid in the media by phytase contributed to the pH changed after the fermentation (Kholed et al., 2020). Table 1 and Table 2 shows the pH values taken at three points during batch culture using peptone and yeast extract as nitrogen source. The initial pH values for all culture was pH 5.5. In peptone fermentation, the pH values of the culture changes slightly into acidic with strain A (4.87), B9 (3.24), L3 (3.19) and RT (3.24). While in yeast extract fermentation, the culture changed into acidic medium with strain A (2.9), B9 (2.46), L3 (2.37) and RT (2.41).

Table 1 and Table 2 shows the reduction in pH values due to the more release of phytase and phosphates contribute to mobilization of more unavailable phosphate. pH values reduction with time of the culture is due to the release of different organic acids such as malate, citrate and oxalate by the microorganisms (Yadav & Tarafdar, 2003). The reduction in pH indicated that there was presence of phosphate solubilizing bacteria in the culture medium that solubilizes phytic acid into inorganic phosphate and produce organic acids in the medium and resulted in acidification of the medium (Hosseinkhani & Emtiazi, 2010). According to Singh et al. (2013) when the pH is lower or higher than the optimum pH, it will affect phytase production as the bacteria producing phytase are sensitive to pH changes in the environment.

Table 1. pH values taken at three points during batch culture using peptone as nitrogen source.

Time (Hour)	A	B9	L3	RT
0	5.50	5.50	5.50	5.50
24	4.55	3.38	3.36	3.34
72	4.87	3.24	3.19	3.24

Table 2. pH values taken at three points during batch culture using yeast extract as nitrogen source.

Time (Hour)	A	B9	L3	RT
0	5.50	5.50	5.50	5.50
24	4.20	4.35	4.00	4.70
72	2.90	2.46	2.37	2.41

Phytase activity

Growth and phytase production of phytase bacterial strains due to the effect of peptone and yeast extract shown in Fig. 2. Usage of yeast extract in fermentation resulting in strain L3 with the highest phytase production (0.216 U/mL) followed by strain B9, RT and A. While in peptone fermentation, RT shows the highest phytase production (0.036 U/mL) followed by strain B9, A and L3. Highest growth in yeast extract fermentation was strain L3, while strain RT shows the highest growth in peptone fermentation compared to other strains.

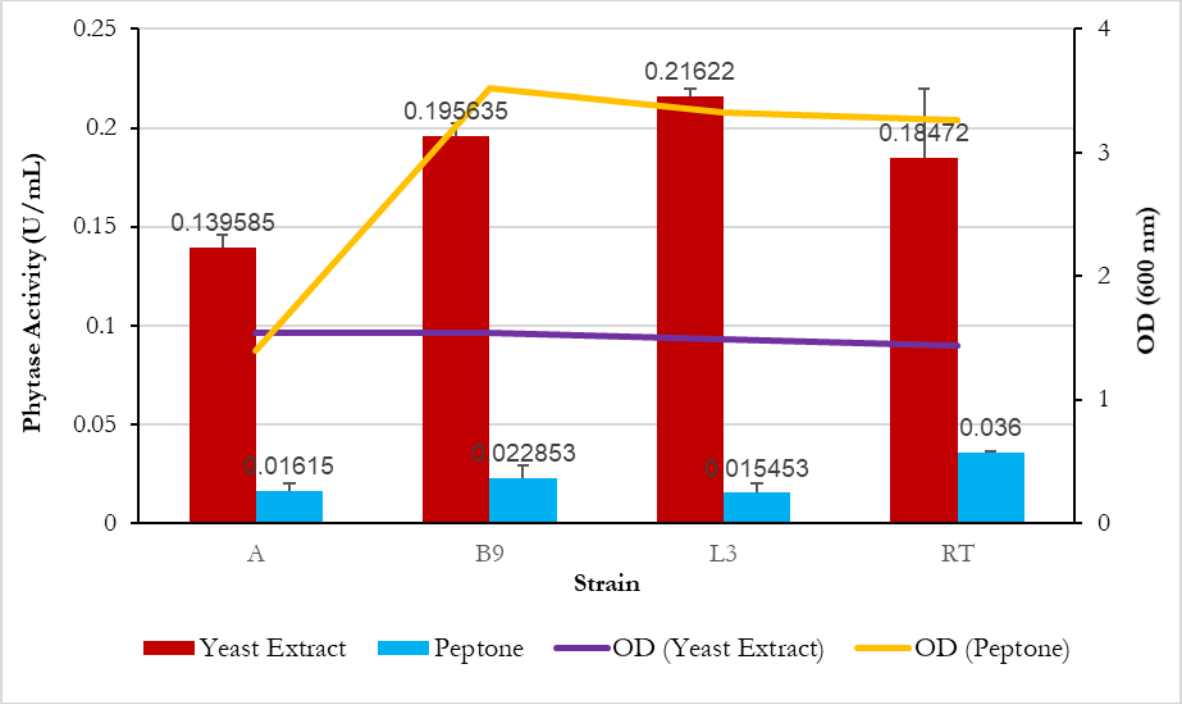

Fig. 2. Effect of nitrogen source on the growth and phytase production. Results are means of two duplicate samples. Bar corresponds to standard deviation.

Fig. 2 shows negative correlations between production of phytase and the growth of four phytase bacterial strains in that two different nitrogen sources. Strain L3 shows the highest phytase production in yeast extract fermentation, while the highest growth was strain B9. In peptone fermentation, strain RT shows the highest phytase production, while the highest growth was strain A. This result shows negative correlation between the growth of cells and phytase production which proved that the production of phytase does not determined by the higher growth of the bacterial cells. A major distinct between phytase production in yeast extract and peptone fermentation clearly shown in Fig. 2. From the result obtained, the enzyme activity was significantly different between the two sources of nitrogen for all bacterial strains, with *P* value of RT (P=0.0253), L3 (P=0.0004), B9 (P=0.0014) and A (P=0.0019).

All bacterial strain produced higher phytase in yeast extract fermentation compared to the amount of phytase production in peptone fermentation. This finding shows that yeast extract is the optimum and best nitrogen source for maximum phytase production as supported by previous studies (Dharmsthiti et al., 2005; Soni & Khire, 2007; Bajaj & Wani, 2011; Kumar et al., 2012; Sasirekha, 2012; Tungala et al., 2013; Singh et al., 2013). Furthermore, Fig. 2 clearly shows that the growth of all bacterial cells was not directly influenced the phytase activity. As shown in Fig. 2, strain L3 in yeast extract fermentation produced highest phytase (0.216 U/mL) even though it has roughly similar OD growth compared to other strains. Strain B9 shows the best growth compared to the other three strains and produced second highest of phytase production (0.196 U/mL).

This result proved that every bacterial strain had different favour in term of nitrogen sources. Yeast extract produced better phytase production (Singh et al., 2013). According to Soni & Khire (2007), yeast extract, peptone and tryptone stimulated maximum phytase production. Tungala et al. (2013) reported that yeast extract with wheat bran as carbon source showed maximum phytase activity as the mixture of yeast extract and wheat bran stimulate production of phytase and increase growth of *Pseudomonas fluorescens*. While in *Aspergillus niger*, peptone as the nitrogen source resulting in maximum yield of phytase production. 1% peptone act as the best source for phytase production (4.7 U/mL) followed by yeast extract (3.5 U/mL), and addition of peptone to the medium resulted in significant increase in phytase activity as compared with medium without nitrogen (Kumar et al., 2012).

CONCLUSION

In conclusion, all the findings obtained in this study to determine the effect of nitrogen sources in phytase production shows that yeast extract act as the best and optimum nitrogen source that contribute to maximum yield of phytase production compared to the usage of peptone in Phytase Screening Medium. Results obtained from this study shows that yeast extract is the optimum nitrogen source as supported by many previous studies. From all the bacterial strains, strain L3 (from Labis, Johor) shown maximum phytase activity in yeast extract fermentation. The objectives of this study were fulfilled in identifying the effects of nitrogen sources used on phytase production. Appropriate nitrogen source greatly affects phytase production. Growth and phytase production were greatly affected in growth medium without nitrogen source. Last but not least, further work in biochemical research and molecular identification is needed to identify molecular identity of the strains used in this study.

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