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Effect of Carbon Sources on Different Strains of Phytase-Producing Bacteria Isolated from Malaysia's Hot Spring

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ABSTRACT

Animal feed from cereal grains and oilseed meals mainly containing phytic acid which has adverse effects on animal nutrition and its environment. Ruminants can easily digest the phytic acid as they have fungi and bacteria in their guts which can produce phytase to degrade the phytic acid. Meanwhile, phytic acid in non-ruminant animals is poorly digested due to the lack of sufficient phytase in their guts. Thus, the feed must be supplemented with inorganic phosphate to ensure it can absorb adequate nutrients. This study aimed to determine the effects of using different carbon sources to the growth of different strains of phytase producing bacteria based on optical density (OD), colony forming unit (CFU), and their phytase production. All four strains of potentially producing-phytase bacteria have been isolated from several hot springs in Malaysia. The bacteria were grown in modified Phytase Screening Medium (PSM) with glucose and lactose as a carbon source and under optimum culture conditions (pH 5.5, 37˚C, 200 rpm) for 72 hours. For quantitative screening of phytase production, the bacterial cultures were harvested to obtain the supernatants that were used to measure the amount of inorganic phosphorus released by the bacterial strains. Among these carbon sources, glucose has shown consistency between their CFU counts and the observed ODs whereas lactose shown inconsistency. Meanwhile, the maximum phytase activity was recorded for all strains in the presence of glucose in which bacteria strain L3 (0.0404 U/mL), RT (0.0359 U/mL), B9 (0.0262 U/mL), and A (0.0263 U/mL). As for the overall, strain L3 (Labis, Johor) gave a promising rate of inorganic phosphate released with optimum phytase activity value of 0.0404 U/mL in presence of glucose and lactose. The optimisation of the fermentation medium can contribute to more economical production of industrial enzyme as phytase has the potential to produce feed additives for poultry feeding.

Keywords: Carbon sources, phytase-producing bacteria, hot spring, phytic acid, phytase, bacteria growth

INTRODUCTION

Phytic acid (myo-inositol hexakisphosphate) or known as phytate in a salt form is a major storage form of organic phosphorus in cereals, legumes, nuts, and oilseeds which comprising 1 to 5 % of their weight (Vats &

Banerjee, 2004; Santos et al., 2004). Although these crops are good sources of essential nutrients, they are also containing high levels of phytic acid which played a role as an anti-nutritional factor. These complexes formation in the intestinal tract will prevent absorption of minerals. Moreover, phytic acid also inhibits particular digestive enzymes.

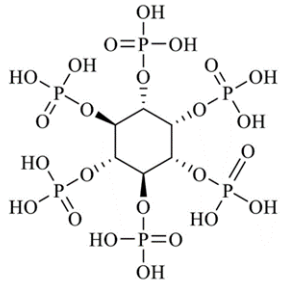

Fig. 1. Structure of phytic acid (IP6, IUPAC)

Phytase is a natural enzyme that can hydrolyse the complex organic forms of phosphorous such as phytate into simpler forms by breaking down the phosphate ester bonds and the utilizable form of inorganic phosphorous is released (Shin et al., 2001; Shivange et al., 2010). Phytases are widespread in nature and they are among plants, animal tissues and microorganisms like bacteria, fungi and yeast (Yao et al., 2011). However, fungi are the most common microorganism that producing phytase (Mullaney & Ullah, 2003). The phytases can be classified into four classes. These four classes are β-propeller phytases (BPPs), cysteine phosphatases (CPs), histidine acid phosphatases (HAPs), purple acid phosphatases (PAPs). BPPs were found in *Bacillus* and some other bacteria whereas CPs usually found in *Selenomonas ruminantium*. Microbes and plants can produce HAPs. As for PAPs, are produced by animals, plants and fungi (Lim et al., 2007).

As undigested phytic acid in animal feeds is excreted by non-ruminant animals in form of feces into rivers and seas causing severe phosphorus pollution to the water resources (Mullaney et al., 2000). Similarly, humans also cannot utilize the phytate that contains in plant-based food products because no phytase enzyme is available in the human intestine. Consequently, many mineral ions are unavailable for human consumption (Fredlund et al., 2006; Kumar et al., 2010b). Therefore, phytase is added during food processing and consumption in order to reduce the adverse effects of phytate in plant-based food products (Kumar et al., 2010b).

High production of phytase is influenced by the optimization of various nutritional and physical parameters including carbon, nitrogen and mineral sources, the composition of the growth medium, pH, temperature, methods of cultivation, the concentration of inoculum, time of incubation, the type of strain, and the cell growth (Lambrechts et al., 1993; Gargova & Sariyska, 2003). Carbon source and nitrogen source give significant effects to the growth of bacteria and enzyme production (Sreedevi & Reddy, 2012). Therefore, creating a suitable medium is essential for the growth of organisms vis-à-vis yield of enzyme (Gao et al., 2009). The optimum conditions for the isolated phytase producing strains were standardized using Phytase Screening Medium (PSM) (Choi et al., 2001). To reduce cost, it is required to utilize a medium composed of low-cost raw materials. Thus, a new formulated medium was prepared based on previously described medium PSM (Kerovuo et al., 1998). Glucose is commonly utilized by microorganisms due to the simplest carbon source which enhances biomass along with the high production of phytase (Das & Ghosh, 2014) as it is providing a faster growth rate compared to other sugars (Monod, 1942). Moreover, Sharma & Shukla (2019) have carried out a study to determine the optimum temperature and pH for maximum phytase production. The optimum temperature and pH are at 37°C (1.32 U/mL) and pH 5.5 (0.82 U/mL) respectively.

Phytase activity was determined by measuring the amount of phosphate released from sodium phytate during enzymatic reaction using the ammonium molybdate method (Engelen et al., 2001). The phytase activity was determined spectrophotometrically at 415 nm using the standard curve prepared from KH_2PO_4. The phytase activity is expressed in phytase units (U). One unit of phytase activity is defined as the amount of enzyme that releases 1 micromole of inorganic phosphorus per ml per minute under the assay condition.

MATERIALS AND METHODS

Bacterial Strains and Overnight Culture

The phytase producing bacterial strains (RT, L3, B9 and A) which collected by Mohamad et al. (2012) were originally from different hot spring in Malaysia which were Dusun Tua (Selangor), Labis (Johor), Ranau (Sabah) and Ulu Lenggong (Kedah), respectively. Each bacterial strain from glycerol stocks was grown on Nutrient Agar (NA) plates. The plates were then incubated at 37 °C for 24 hours. By using an aseptic technique, the single colony formed on the streaked NA plate was inoculated into a sterile 50 mL Falcon tube containing 10 mL of Luria Bertani (LB) broth by using an inoculation loop. The mixtures were then incubated in an incubator shaker (37°C, 180 rpm) for at least 18 hours. All single strong colonies were selected and maintained in slant cultures.

Screening for Phytase Producing Bacteria Strains

The screening for phytase producing bacteria strains was carried out according to Alias et al. (2018) where the single colonies from Nutrient Agar (NA) plates were streaked on Phytase Screening Medium (PSM) agar which containing (g/L); 20.0 glucose, 4.0 sodium phytate, 2.0 calcium chloride, 5.0 ammonium nitrate, 0.5 potassium chloride, 0.5 magnesium sulphate, 0.01 iron sulphate, 15.0 agar. The PSM was adjusted to pH 5.5 and sterilized using an autoclave (15 psi, 121°C, 20 min). Next, the plates were incubated at 37°C for 96 hours.

Preparation of Phytase Screening Medium

Carbon sources used were glucose and lactose at a concentration 1.0 %. The Phytase Screening Medium (PSM) was prepared according to Dharmsthiti et al. (2005) which contained 10 g carbon source, 0.5 g potassium chloride, 0.5 g magnesium sulphate, 5 g ammonium nitrate, 0.1 g manganese sulphate, 5 g calcium chloride, 0.1 g iron sulphate, 1 g citric acid, 1 g yeast extract, 1 mL Tween-80, 80 mL soybean extract solution and filled with distilled water up to 1 L.

The soybean extract was prepared by soaking 100 g of 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. The PSM then was adjusted to pH 5.5 by the addition of 1 M NaOH. Next, 100 mL of PSM was transferred into two different 500 mL conical flasks. The flasks were sterilized by autoclaving (15 psi, 121 °C, 15 min).

Batch Culture

A volume of 1 mL of overnight culture was inoculated into each 500 mL flask containing 100 mL of Phytase Screening Medium (PSM) under aseptic technique. The flasks then were incubated in an incubator shaker (37°C, 200 rpm) for 72 hours. Next, the optical density (OD) of the bacterial culture was read at 600 nm.

Growth Analysis

The wavelength of the spectrophotometer was set to 600 nm. 1 mL of bacterial culture was pipetted out periodically at every hour intervals for the first 8 hours and 24 hours and then was transferred into a new cuvette. Next, the absorbance of the bacterial culture was measured on Shimadzu UVmini-1240 UV-Vis

Spectrophotometer at 600 nm wavelength. If the optical density (OD) was larger than 0.8, the dilution would be done by using phosphate-buffered saline (PBS) solution.

Sampling for Colony Forming Unit (CFU) was done at 1, 5 and 24 hours of incubation. A volume of 100 µL of bacterial culture was serially diluted with 900 µL of sterile phosphate-buffered saline (PBS) solution up to 10^{-7} dilution under aseptic technique. 100 µL of diluted bacterial culture then was inoculated on nutrient agar (NA) plates by using the spreading method. Next, the plates were incubated in an incubator at 37°C for 24 hours. The number of colonies formed was counted by using the colony counter. The pH of the bacterial culture was measured using a pH meter at 0, 24 and 72 hours of incubation.

Phytase Assay

The phytase activity was assayed according to Rocky-Salimi et al. (2016) method. The bacterial cultures were harvested by centrifugation (4°C, 12 000 rpm, 20 min). The supernatants obtained were used for phytase assay. 400 µL of the reaction mixture of 1.5 mM sodium phytate in 100 mM Tris–HCl buffer (pH 7.0) was added into a microcentrifuge tube containing 100 µL of crude enzyme. The mixture then was incubated at 55°C for 30 minutes. The reaction was stopped by adding 400 µL of color reagent solution (150 µL of 0.24 % ammonium vanadate, 150 µL of 10 % ammonium molybdate, 100 µL of 65 % nitric acid) and the samples were centrifuged (25°C, 15 000 g, 10 min). Next, the optical density (OD) was read at 415 nm and compared with the standard curve prepared from $KH_2\ PO_4$.

Statistical Analysis

In cell culture, the measurement of parameters such as biomass (OD and CFU), pH, and phytase activity was optimized based on the type of carbon sources used, which were glucose and lactose. Results data were analysed by using Microsoft Excel Software 2016 and were presented with ± standard deviation; tested via T-test to determine the *P* value. The means of the group were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Growth Analysis

In this study, glucose and lactose were used for carbon sources optimisation. A study conducted by Qasim et al. (2017) for the effect of carbon sources on the phytase production by *Aspergillus tubingensis* SKA shows that the addition of glucose produced the highest phytase yield (42.0 U/mL) while lactose yielding significantly less phytase. Similar data showed for *Penicillium purpurogenum*, in which among different carbon sources used;, corn starch, glucose maltose, lactose and sucrose, the study reported only glucose achieved the maximum phytase productivity (Awad et al., 2014).

Glucose is commonly utilized by microorganisms due to its simplest carbon structure which enhances biomass along with high production of phytase (Dash & Gosh, 2014). It provides so much energy to the cells because it directly entered glycolysis in form of glucose 6-phosphate and is consumed via the tricarboxylic acid (TCA) cycle (Ronimus & Morgan, 2006; Deutscher et al., 2006). Based on Fig. 2, bacterial strains grown in PSM media supplemented with lactose have higher OD compared to the bacterial cultures grown in glucose as each point of an hour showing a different value in the ODs. Out of all bacteria strains, strains A and B9 were found to be better strains that grow in the media supplemented with lactose and glucose. Between these carbon sources, all of the strain shows more favourable growth conditions in lactose compared to glucose.

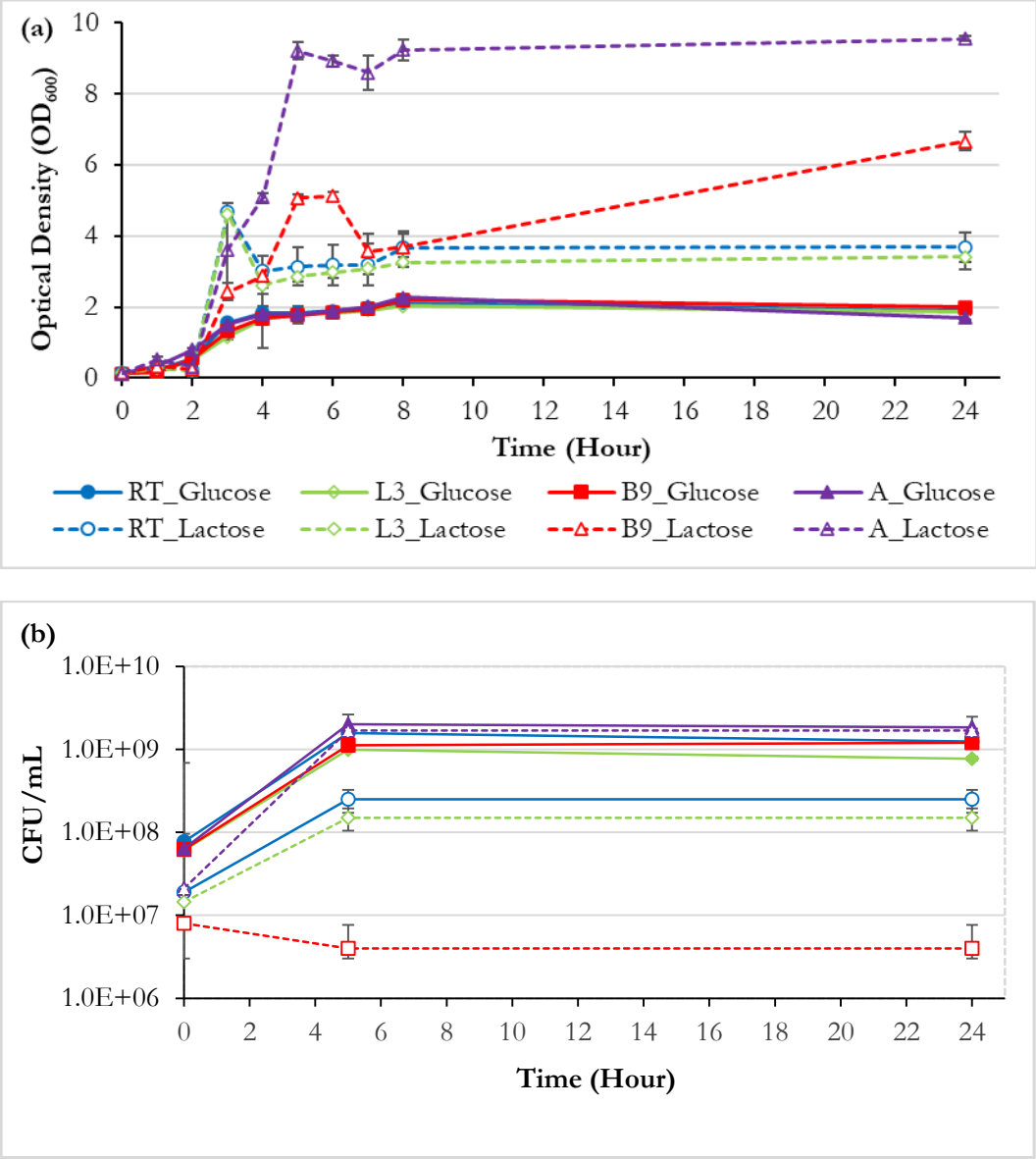

Fig. 2. Correlation of all strains growth for the study of carbon sources optimisation for phytase production, evaluated by (a) OD_{600} measurement, and (b) CFU/mL counting. All data shown are mean values from replica flasks with error bars are ±1 standard deviation.

Values of the OD were not significantly different between glucose and lactose for RT and L3 strains according to the least significant difference at $P<0.05$, in which strain RT ($P=0.052$) and L3 ($P=0.055$) showed a slower rate of growth. While for strain B9 and A, they were found to be the superior strains that grew in the PSM media, in which the OD value were significantly different, with B9 ($P=0.043$) and A ($P=0.008$). However, some of the CFU counts were surprisingly inconsistent with the observed ODs when the bacterial strains were grown in the PSM media supplemented with lactose. Strains L3, B9 and RT give out lower viable counts compared to strain A (2.0×10^9 CFU.mL^{-1}), where it is recorded the highest value from all strains at two-time points (5 and 24 hours). As for bacterial strains supplemented with glucose have shown consistency between their CFU counts and the observed ODs, where the highest viable count was recorded by strain A (1.27×10^9 CFU.mL^{-1}) and strain RT (1.2×10^9 CFU.mL^{-1}) gives out the lowest values.

A study was conducted by Cheng et al. (2015) for the fermentation process of *Streptococcus suis,* where the carbon sources used were glucose, sucrose, lactose and galactose. In contrast the the observation above, the lowest cell density (0.884) and viable count (1.42×10^9 CFU.mL^{-1}) was recorded by glucose. Meanwhile, in the presence of lactose, the growth yield is increased in both cell density (1.023) and viable count (1.72×10^9 CFU.mL^{-1}).

pH Analysis

At the end of incubation hours, all bacterial strains shown decreasing pH (Fig. 3), in which bacterial strains RT (2.41), L3 (2.37), B9 (2.46) and A (2.90) incubated in glucose gives out the lower pH compared to lactose. The previous study showed that acidic conditions can promote microbial growth (Badamchi et al., 2013) and enzyme activity (Moreira et al., 2014). This is proved by strain A which has grown in a medium containing lactose was recorded an alkaline pH (7.28) and resulting from the lowest phytase activity. Roy et al. (2012) have reported that increasing pH will influence the active site, consequently, enzyme activity decreased vis-à-vis enzyme-substrate complex formation. Therefore, the higher pH, the lower the phytase activity.

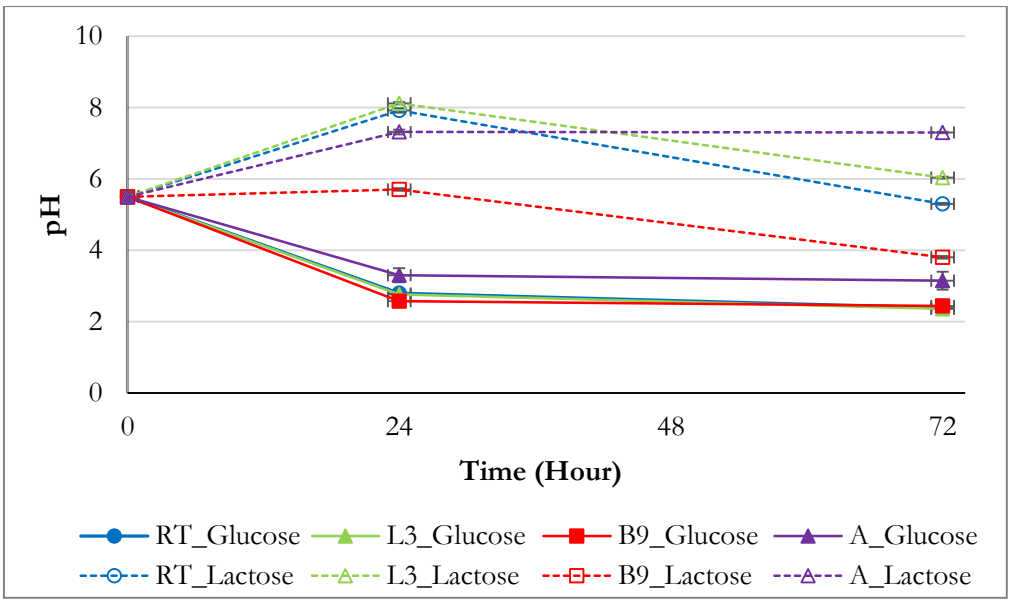

Fig. 3. pH changes of bacterial cultures at 0, 24 and 72 hours of incubation.

Phytase Analysis

After 72 hours of incubation at 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 (Engelen et al., 2001). The phytase activity is expressed in phytase units (U). One unit of phytase activity is defined as the amount of enzyme that releases 1 micromole of inorganic phosphorus per mL per minute. From the result obtained, there was no significant difference for enzyme activity between the two carbon sources for most bacterial strains, with *P* value of RT (P=0.277), L3 (P=0.259), and A (P=0.256). However, strain B9 showed significant different with (P=0.037).

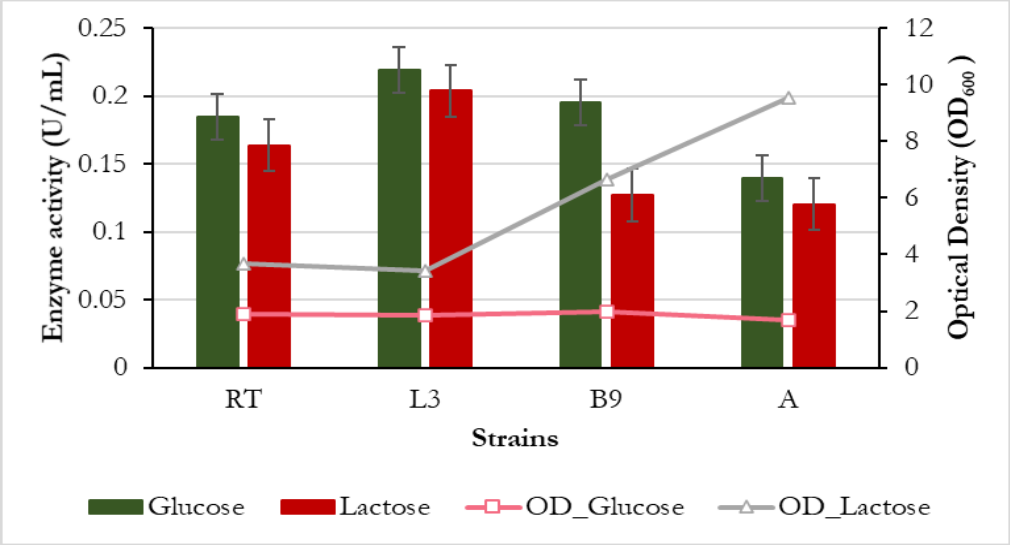

Fig. 4. Effect of glucose and lactose on growth and phytase production of phytase bacterial strains. Results are means of two duplicate samples. Bar corresponds to standard deviation.

Based on Fig. 4., all phytase bacterial strains have shown their ability to produce phytase enzyme in both glucose and lactose. Among these carbon sources investigated, the maximum phytase activity was recorded for all strains in the presence of glucose in which bacteria strain L3 (0.0404 U/mL), RT (0.0359 U/mL), B9 (0.0262 U/mL), and A (0.0263 U/mL). Meanwhile, the minimum phytase activity was recorded in presence of lactose for most observed strains, where strain A (0.0120 U/mL) and B9 (0.0127 U/mL) gave out the lowest values. This indicated that the strains are preferred to produce higher phytase when presence of glucose as the carbon source compared to lactose. Previous study showed that lactose is poor carbon sources for enzyme production since it is not induced phytase activity compared to glucose (Khianngam et al. 2017). Moreover, the highest phytase production was contributed by the bacteria strain L3. This study proved the statement made by Alias et al. (2017) whose stated that strain L3 is the best phytase producer when using glucose as the carbon source.

CONCLUSION

The appropriate carbon source for the optimum phytase production of different phytase producing bacteria strains has successfully determined. In addition, the growth curve of different phytase-producing bacterial strains was also successfully identified based on the results of optical density (OD). Among the two carbon sources investigated, the maximum phytase activity was recorded in presence of glucose. From all the bacterial strains, bacteria strain L3 (strain from Labis, Johor) shown maximum phytase activity in both glucose and lactose. In the future, biochemical research and molecular identification may also be carried out to identify molecular identity in the strains. This study can provide an efficient strategy to produce maximum phytase as many 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.

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