Phytase Production by *Lactobacillus plantarum* A1-E in Submerged and Solid-State Fermentation

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ABSTRACT

Enzyme activity is influenced by several important factors, including the amount and type of substrate, solvent type, pH, temperature, presence of inhibitory and activating ions, and concentration of enzymes. Therefore, this research aimed to evaluate phytase production from *Lactobacillus plantarum* A1-E using submerged (SmF) and solid-state fermentation (SSF). Phytase production was determined using SmF with fructose and sucrose as the primary carbon sources at concentrations of 4.5%, 6%, and 7.5%. Additionally, SSF was conducted using three distinct substrates, including soybean Meal, rice Bran, and pollard. The results indicated that the highest phytase activity was achieved through SSF when rice bran was used as a substrate (88.48 U/mL or 4.65 U/mg). The use of 4.5% sucrose as a carbon source in the SmF technique showed the highest specific phytase activity (4.38 U/mg) compared to other carbon sources at various concentrations. The addition of metal ions showed that Fe^{2+}, Mn^{2+}, and Co^{2+} at concentrations of 1-5 mM, Mg^{2+} and Zn^{2+} at concentrations of 3-5 mM, and Ca^{2+} at a concentration of 3 mM acted as activators that increased phytase activity compared to control. Meanwhile, Mg^{2+} and Zn^{2+} at concentrations 1-2 mM were inhibitors.

Keywords: Phytase, *Lactobacillus plantarum*, submerged, fermentation, solid-state fermentation

INTRODUCTION

Monogastric livestock are deficient in the phytase enzyme required for phytate digestion. This enzyme is particularly relevant as phytate-P is the primary form of the essential nutrient phosphorus (P) found in grains (McKinney et al., 2015). Phytate hydrolysis by phytase was required to release phosphate from the phytate-P binding (Suryani et al., 2021). Phytase is a class of enzymes that hydrolyze the biochemical degradation of myo-inositol hexakisphosphate (phytic acid), leading to the liberation of myo-inositol and inorganic orthophosphate or free phosphorus (Mehak et al., 2019). Animals, plants, and microbes, such as yeast, bacteria, and fungi, are the most common sources (Penidez et al., 2020), with phytases from microbes being more commercially viable (Singh and Satyanarayana, 2011). Bacterial-derived phytases offer benefits, such as efficient phosphorus release and resistance to protease. In addition, phytate-degrading enzymes from Gram-positive bacteria are typically extracellular (Mehak et al., 2019).

In recent years, many phytase-producing lactic acid bacteria (LAB), including *L. fermentum* (Sharma

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et al., 2018), *L. sanfranciscensis* (De Angelis et al., 2003), *L. pentosus* (Palacios et al., 2005), (Amritha et al., 2017), and *L. coryniformis* (Demir et al., 2018) have been isolated from various sources and purified. Diverse LAB strains are used for phytic acid hydrolysis through the use of enzymes isolated from microorganisms or post-culture medium. This LAB produces an effective phytase *in-situ* for the production of feed or fermentation (Kłosowski et al., 2018).

Microorganisms serve as hosts for both submerged and solid-state fermentation in the commercial production of phytases, including bacteria, yeasts, and fungi (Handa et al., 2020). Submerged Fermentation (SmF) is a form of fermentation in which microorganisms grow in an enclosed liquid medium containing various nutrients that are dissolved to suspend particulate materials or within a commercial medium used in a shake flask (Handa et al., 2020). Solid-state fermentation (SSF) is a technique that involve the microorganisms growth on solid medium without an excess of liquid (Vandenberghe et al., 2021).

Biological and physicochemical mechanisms influence phytase activity. The initial processes lead to enzyme changes on synthesis rates, shifts in isoenzyme production, and modifications in composition of microbiome community, while the latter processes lead to changes in reaction of absorption–desorption, the rates of substrate diffusion, and the rate of enzyme degradation (Wallenstein et al., 2003). The quantity and substrate type (Gaind et al., 2015), the solvent type, temperature, pH, inhibitor and activator ions availability, and dosage of enzymes and products are all essential parameters that influence enzyme activity (Azeem et al., 2015).

Various agricultural waste products have been widely used as alternatives to poultry feed ingredients. Agricultural waste, such as soybean meal, rice bran, and pollard are rich in vital nutrients and minerals, making them suitable for animal feed, with utilization reaching as high as 90% (Sukma et al., 2021). Seidavi et al. (2021) reported that a high phytate content was found in some agricultural wastes used as poultry feed ingredients, accounting for 60-80% of total phosphorus. The SSF method allows for the use of agricultural waste with high phytate concentration as substrates for phytase production, increasing the phytase activity obtained.

Sumengen et al. (2013) stated that the different fermentation methods and supplementation with various minerals greatly influenced the phytase activity obtained from *L. plantarum*. In addition, Shivanna and Venkateswaran (2014) conducted research comparing fermentation methods (SMF and SSF) using various types of substrates to enhance the phytase activity of *Aspergillus ficuum* SGA 01 and *A. niger* CFR 335. The effect of different fermentation methods with various carbon sources and mineral supplements on the phytase activity produced has been evaluated by several other researchers (Singh et al., 2013); (Thyagarajan et al., 2014; Abedi et al., 2019; Suliasih and Widawati, 2019).

Industries prioritize large-scale fermentation procedures using affordable feedstocks to reduce the cost of phytase production. Sucrose and sugarcane molasses are frequently used in protein and biochemical synthesis due to their low-cost and easy fermentability (Boonchoo et al., 2019). The use of protein-rich substrates in SSF provides carbon, vitamins, and minerals (Thyagarajan et al., 2014). In addition to being used as SSF substrates, the byproducts of rice bran and wheat bran are widely known for being potential substrates for phytase synthesis due to their nutrient content (Roopesh et al., 2006). Soybean meal is also commonly as solid medium for SSF due to its high nutritional value particularly protein and relatively low cost (Su et al., 2018).

To date, optimization methods for phytase production from microorganisms are still being explored due to the numerous factors involved in the phytase production process required to obtain optimal phytase activity. Therefore, this research was conducted to evaluate SmF (in various types and concentrations of carbon sources) and SSF (on various substrates in producing phytase from *Lactobacillus plantarum* A1-E.

METHODS

Microorganisms and Preparation of Inoculum

Lactobacillus plantarum A1-E, isolated from the ileum of native chickens, was used as a phytase-producing microorganism. The process started by refreshing the *L. plantarum* A1-E isolates from the stock culture on selective de Man, Rogosa, and Sharpe (MRS) broth with a media concentration of 52 g/L. The ratio of *L. plantarum* A1-E to media was 1% (v/v). Subsequently, the inoculated media were incubated at 37 °C for 24 hour. After refreshment, the total plate count of *L. plantarum* A1-E isolates was determined to obtain a population of 10^7-10^8 CFU/mL. The culture was registered in the Indonesian Culture Collection with the collection number InaCC B1550.

Preparation of Phytase Production Medium

The medium for the SmF method was prepared according to the modified method by Thyagarajan et al., 2014 (Table 1). The medium preparation for the SSF method began with three types of substrates, namely soybean meal, rice bran, and pollard, that were blended and filtered through a 40 mesh filter (approximately 1 mm

particle size). After filtering, the SSF substrate was weighed (10 g) and placed in Erlenmeyer flask, then distilled water (10 ml) was added. The flask was covered with gauze and aluminum foil, then sterilized using an autoclave at 121 °C for 40 minutes and a pressure of 15 psi.

Phytase production Using the SmF Method

The fermentation medium (100 mL) was prepared in Erlenmeyer flask and sterilized in autoclave. Two types of carbon sources with three levels of concentration could be seen in Table 2. The carbon sources were filtered, sterilized in autoclave, and added to a fermentation medium. Using aseptic techniques, 0.1 mL (10^8 CFU/mL) of the isolate suspension was introduced. Furthermore, the inoculated medium was stored in an shaker incubator at 150 RPM at 20-25 °C for 5 days of incubation. After incubation, the inoculated medium was filtered using a muslin cloth, and the obtained filtrate was separated with centrifugation at 10000 RPM at 4 °C for 10 minutes. The supernatant obtained was collected as a phtytase crude enzyme (Thyagarajan et al., 2014).

Table 1. Standard media fermentation

No.	Chemicals	Concentration (g/L)
1.	Starch	50
2.	Glucose	25
3.	Sodium Nitrate	8.6
4.	Potassium dihydrogen phosphate	0.04
5.	Potassium Chloride	0.5
6.	Magnesium Sulfate	0.5
7.	Ferrous sulfate	0.1

Table 2. Two carbon sources with three concentration levels

No.	Carbon source	Concentration (%)
1.	Fructose	4.5, 6.0 and 7.5
2.	Sucrose	4.5, 6.0 and 7.5

Phytase Production by SSF

The enzyme production process by the SSF method was carried out based on the modified method by Mandviwala and Khire (2000). After sterilization, the SSF substrate was cooled to approximately 20-25˚C. A 10% suspension of *L. plantarum* A1-E liquid isolate was inoculated into the substrate and then homogenized with a glass stirrer. The mixture was placed in an incubator at 37˚C for 48 hours. After the incubation, a 150 mL solution of 2% $CaCl_2.H_2O$ solution was added to the inoculated substrate in a 1:5 ratio and then shaken using a rotary shaker at a rotation speed of 200 rpm for two hours at 20-25˚C. After shaking, the inoculated substrate was filtered using two layers of muslin cloth. The filtrate was then separated using a centrifuge at 4000 rpm at 4°C for 30 minutes. The resulting centrifuged supernatant obtained was collected as a phytase crude enzyme.

Phytase Activity Assay

The activity of phytase was measured according to the method outlined by Vohra and Satyanarayana, 2001. In this procedure, 1 mL of enzyme was added to 1.5 µM of sodium phytate (dissolved in 0.1 M acetate buffer, pH 4) then 0.5 mL of 0.1 M acetate buffer (pH 4) was added to the mixture. The reaction was homogenized and incubated at 60 °C for 15 minutes. Subsequently, 2 mL of 10% TCA was added to the reaction mixture and homogenized. Preparation of 1 mL of a colorant solution was carried out, consisting of 2.7% ferrous sulfate solution and 1.5% H_2SO_4 dissolved in 5.5% ammonium molybdate in a 1:4 ratio and placed at room temperature for five minutes (Suryani et al., 2021). A standard calibration curve was prepared with a 50-500 µM concentration by dissolving 6.8 mg of KH_2PO_4 in 0.1 L of acetate buffer (0,1 M, pH 4). A unit of phytase is described as the amount of enzyme that liberates 1 µmol inorganic phosphate per mL per minute under specific conditions (Qasim et al., 2016).

Specific Phytase Activity Assay

Specific phytase activity was used to evaluate the ability of phytase to hydrolyze a substrate per mg of protein dissolved in solution (Damayanti et al., 2017). Dissolved protein levels in phytase were measured using the Lowry et al. (1951) method. This involved mixing 0.5 mL of Crude phytase with 0.5 mL of Lowry B reagent consisting of 1% $CuSO_4.10H_2O$, $C_4H_4KNaO_6.4H_2O$ 2%, 5N NaOH, and 4% Na_2CO_3 in 5N NaOH. The mix solution was homogenized and stored at room temperature for 15 minutes. Subsequently, 1.5 mL of Lowry A reagent (10% Folin-Ciocalteu's reagent) was added to the reaction mixture and placed at room temperature to complete the reaction for 45 minutes. The absorbance values of both samples and standard were read using a spectrophotometer at a wavenumber of 700 nm. The standard solution was prepared by dissolving 1.5 mg of Bovine Serum Albumin (BSA) in 50 mL of distilled water. This resulted in a standard protein concentration that was adjusted to 0.3 mg/mL (Suryani et al., 2021).

Effect of Metal Ions on *L. Plantarum* A1-E Phytase Activity

The inhibitory or stimulatory effect of metal ions on the phytase activity of *L. plantarum* A1-E was performed by dissolving enzyme solution and 1-5 mM (final concentration) of metal ions was incubated at 37ºC for 1 hour. Subsequently, citrate buffer (100 Mm, pH 3.4) containing 2 Mm sodium phytate as substrate was added, and the activity of phytase was measured under standard conditions (Sumengen et al., 2013).

Statistical Analysis

All the experiments were conducted in triplicate. The averages and standard deviations were recorded. Data were analyzed statistically, and the differences between average values were evaluated using the analysis of variance (ANOVA). In addition, Duncan's posthoc test was applied to evaluate the differences among mean treatments (Gomez and Gomez, 2010). Statistical analyses were carried out using Costat software (Cohort, 2008). Statistically, $p<0.05$ was considered as significant value.

RESULTS AND DISCUSSION

Effect of Two Types of Carbon Sources with Three Concentration Levels in SmF Method on Phytase Activity

Phytases (myo-inositol hexakisphosphate phosphor hydrolases) hydrolyze the phospho-monoester bonds of phytic acid (phytate) (Jatuwong et al., 2020) and liberate free myo-inositol phosphates and minerals (Suryani et al., 2021). Microbes were used to produce phytase using three distinct culture methods such as solid-state, semisolid, and submerged fermentation (Shivanna and Venkateswaran, 2014). Handa et al. (2020) stated that phytase was produced commercially using microorganisms such as bacteria, yeast, and fungi as hosts using the SmF and SSF methods.

SmF is a process where microorganisms grow within an enclosed liquid medium containing dissolved nutrients divers for suspending particulate particles. This approach is used for commercial types of medium grown in the shaken flask. Several variables impact phytase activity in the SmF method, such as microbial strain, culture conditions, substrate properties, and nutrient availability including carbon and nitrogen sources (Jatuwong et al., 2020). Carbon, as a component of culture media, is essential to the growth and activity of phytase enzymes (Abedi et al., 2019).

The effect of two types of carbon sources with three concentration levels on the production of phytase was shown in Table 3. The result showed that supplementation with 4.5% sucrose in the SmF medium yielded the highest specific phytase activity (4.38 U/mg), followed by 7.5% sucrose supplementation. The result indicated that sucrose was the best carbon source to produce the maximum phytase activity in the SmF technique among the several carbon sources (fructose and sucrose) employed for *Lactobacillus plantarum* A1-E phytase synthesis. This research result was similar to Aigul et al. (2015), who stated that 1% sucrose as a carbon source promoted maximum phytase activity. Similarly, Abedi et al. (2019) showed that sucrose was the best carbon source, with maximum phytase activity of 0.71±U/mL. The use of sucrose led to increased cell production after 24 hours, making it a preferred carbon

Table 3. Effect of two types of carbon sources on phytase production with three concentration levels

Parameter	Carbon sources (%)						*P*-Value
	Fructose			Sucrose			
	4.5	6	7.5	4.5	6	7.5	
Phytase activity (U/mL)	32.78±3.70^{a}	19.19±0.98^{bc}	16.65±0.08^{cd}	18.53±0.11^{cd}	15.29±1.16^{d}	22.30±2.21^{b}	0.00***
Protein concentration (mg/mL)	15.28±0.89^{a}	13.19±0.67^{b}	13.88±1.52^{ab}	4.25±0.30^{d}	6.80±0.04^{c}	7.61±1.05^{c}	0.00***
Specific phytase activity (U/mg)	2.15±0.25^{c}	1.46±0.01^{d}	1.21±0.12^{d}	4.38±0.33^{a}	2.25±0.16^{c}	2.94±0.21^{b}	0.00***
Phytase yield (U/g)	210.58±19.61	203.84±14.89	196.46±0.91	218.62±1.31	180.42±13.65	202.56±2.04	ns

Note: Values are mean ± standard deviation of triplicate analysis.
$^{a–d}$Different letters are significantly different ($p<0.05$).

source for phytase synthesis (Sugiharto, 2018). In recombinant phytase production, sucrose in molasses can act as an OtMal system inducer in *O. thermomethanolica* yeast. Additionally, it can enhance cell formation and phytase activity (Boonchoo et al., 2019).

Effect of Various Substrates in SSF Method on Phytase Activity

SSF is the growth of organisms on moist medium in the absence of excessive liquid. It is more cost-effective than the SmF method due to fewer energy requirements, reduced waste, uncomplicated oxygen transfer, and less bacterial contamination. Several significant agro-industrial wastes, including sugarcane bagasse, orange baggase, cassava bagasse, coffee husk, wheat bran, sugar beet pulp/husk, oil cakes, grape seed, grape juice, and apple pomace have been employed as solid substrates for the SSF process (Vandenberghe et al., 2021). Commercial SSF phytases production commonly involves cultivating phytase-producing bacteria on wheat bran, which gives a surface for microbial to attach and serves as a nitrogen and carbon source (El-Shishtawy et al., 2014).

The SSF technique employing rice bran as the substrate produced the greatest phytase activity (4.65 U/mg) from *L. plantarum* A1-E (Table 4). This result aligned with McKinney et al. (2015), who achieved a maximum phytase activity of 3.5 U/mg from E. coli using wheat bran as a solid substrate under SSF conditions. Previous research indicates that the use of wheat bran was the best for the production of phytase with an activity of 2.5 U/g (El Gindy et al., 2009). Moreover, Roopesh et al. (2006) showed that combining rice bran and various oil cakes as a substrate in SSF resulted in the highest phytase activity (32.2 U/gds) from *M. racemosus*. The nutritionally dense rice bran used as substrates in SSF may be responsible for the increased enzyme production. Rice bran has a high concentration of dietary fibers such as pectin, beta-glucan, and gum, alongside 12-13% oil. It also contains ferulic acid (4-hydroxy-3-methoxy cinnamic acid), which could be a structural component of nonlignified cell walls. The high molecular weight of complex polysaccharides, including cellulose, hemicellulose, lignin, and starch, are found in rice bran which serves as supplementary carbon sources. Additionally, a higher level of total phosphorus reduces phosphate limitation in the culture media (Shivanna and Venkateswaran, 2014).

Effect of Metal Ions at Various Concentrations on Phytase Activity

The Fe^{2+} ion at 5 mM concentration performed the highest phytase activity compared to other evaluated metal ions (285.77%). Of the six types of metal ions tested, at concentrations of 1-5 mM, Fe^{2+}, Mn^{2+}, and Co^{2+} were enzyme activators. Mg^{2+} and Zn^{2+} ions at concentrations of 3-5 mM also acted as activators, enhancing phytase activity beyond the control. At a concentration of 3 mM, Ca^{2+} ions also showed an activator effect. Conversely, Ca^{2+} ions at concentrations of 1, 2. 4, and 5 mM were enzyme inhibitors, followed by Mg^{2+} and Zn^{2+} ions at concentrations of 1-2 mM (Table 5; Figure 1).

Phytases are conditionally sensitive enzymes, and their existence is determined by the substrate molecule impacted by the enzyme which the product formed during the enzymatic reaction, or other precursor characteristics for the enzymatic reaction. Several factors including concentration of enzyme and substrate, pH, temperature, ionic strength, allosteric effects, and also the inhibitors or activators presence are essential factors that affect phytase activity. Sümengen et al. (2012) found that Hg^{2+}, Mn^{2+}, Mg^{2+}, and Cu^{2+} ions stimulated the phytase activity at 5 mM and Fe^{2+} ion stimulated activity at 1 mM, which was consistent with the results of this research. Similarly, De Angelis et ala. (2003) identified Hg^{2+}, Fe^{2+}, Mn^{2+}, $Mg^{2+,}$ and Cu^{2+} ions as activators of phytase production

Table 4. The effect of different substrates on the activity of phytase

Parameter	Substrates			*P-Value*
	Soybean meal	Pollard	Rice Bran	
Phytase activity (U/mL)	21.97±0.83^{c}	30.25±1.81^{b}	88.48±5.49^{a}	0.00***
Protein concentration (mg/mL)	26.83±0.09^{a}	18.53±0.82^{b}	19.01±0.24^{b}	0.00***
Specific phytase activity (U/mg)	0.82±0.03^{c}	1.64±0.15^{b}	4.65±0.26^{a}	0.00***
Phytase yield (U/g)	94.83±3.80^{c}	126.93±6.98^{b}	351.015±26.37^{a}	0.00***

Note: Values are mean ± standard deviation of triplicate analysis.
$^{a-c}$Different letters are significantly different (p<0.05).

Table 5. Effect of metal ions at various concentrations on phytase activity

Metal ion	Residual relative activity (%)				
	1 mM	2 mM	3 mM	4 mM	5 mM
Control	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
$MgCl_2$	39.39±4.76	45.82±3.70	135.09±13.54	152.54±12.72	203.87±12.28
$FeCl_2$	100.15±4.89	226.64±11.74	242.64±3.57	241.27±0.79	285.77±3.44
$ZnCl_2$	95.52±3.31	89.84±1.98	158.44±7.14	147.12±14.64	122.71±1.45
$CoCl_2$	123.90±12.21	108.61±5.68	129.14±8.06	113.32±10.66	113.32±3.70
$MnCl_2$	136.85±14.33	132.80±14.54	187.12±6.74	211.84±8.72	219.46±1.72
$CaCl_2$	57.41±6.74	84.24±11.10	106.40±9.65	86.31±6.21	88.30±3.26

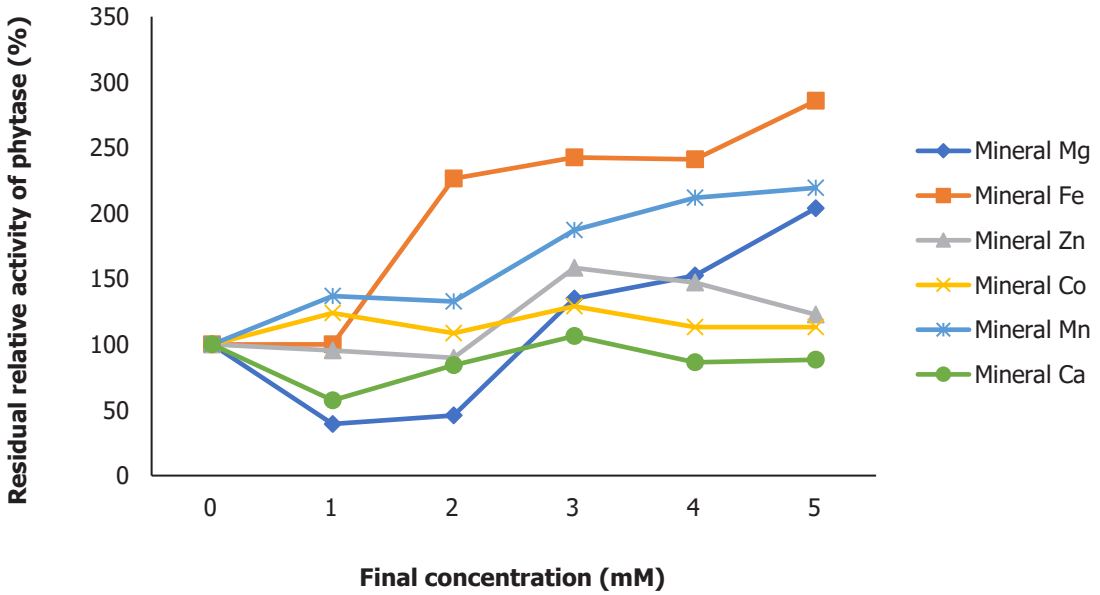

Figure 1. Residual relative activity of phytase from *L. plantarum* A1-E

from *Lactobacillus brevis*. The affinity of certain divalent ions for binding sites in the phytase structure acts as an activator. Different sites with high affinity were found, including two sites at the periphery of the molecule and one site at the central channel. The ion at the central channel cannot chelate with the substrate to form a stable complex, increasing the phytase stability. This stability is perturbed when various divalent metal ions interact, giving rise to the formation of stable metal salts and disrupting substrate-phytase interaction, ultimately inhibiting enzymatic activity. Liu et al. (2021) stated that metal ions in the environment affected the catalytic reaction between phytase and its substrate. Such interaction results in a stable complex, which causes acidic phytase to have limited ability to hydrolyze the phytase-metal complex into inositol and phosphorus. Conversely, different metal ions demonstrate different affinity sites with phytase. When the divalent metal is combined with phytase, it causes enzyme denaturation due to structural changes that lead to reduced catalytic function. Zn^{2+} ions can bind to both enzymes and enzyme-substrate complexes at inactive sites..

CONCLUSION

In conclusion, the SSF method employing rice bran as a solid substrate yielded the highest activity of phytase (88.48 U/mL or 4.65 U/mg). The addition of metal ions showed that Fe^{2+}, Mn^{2+}, and Co^{2+} at concentrations of 1-5 mM, Mg^{2+} and Zn^{2+} at concentrations of 3-5 mM, and Ca^{2+} at a concentration of 3 mM had activator

effects, leading to enhanced phytase activity compared to control. Meanwhile, the metal ions with inhibitor effects were Mg^{2+} and Zn^{2+} at concentrations of 1-2 mM.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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