VOLUME 7 NOMOR 1 JUNI 2020 ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejurnal.bppt.go.id/index.php/JBBI

BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT PHYTASE ENZYME (phyK) FROM *Klebsiella* sp. ASR1 ENCAPSULATED WITH ALGINATE

Karakterisasi Biokimia Enzim Fitase Rekombinan (phyK) dari *Klebsiella* sp. ASR1 Yang Dienkapsulasi Dengan Alginat

Muhammad Eka Hidayatullah[1], Sajidan[1,2]*, Ari Susilowati[1], Baraka Stewart Mkumbe[1], Ralf Greiner[3]
[1]Bioscience Department, Graduate Program, Universitas Sebelas Maret,
Jl. Ir. Sutami 36A Kentingan, Jebres, Surakarta 57126, Indonesia,
[2]Laboratory of Biotechnology, Study Program of Biology, Faculty of Teaching and Educating,
Universitas Sebelas Maret, Jl. Ir. Sutami 36A Kentingan, Jebres, Surakarta 57126, Indonesia
[3]Max Rubner-Institut, Federal Research Institute of Food and Nutrition, Department of Food Technology and Bioprocess Engineering, Haid-und-Neu-Straße 9, 76131 Karlsruhe
*Email: sajidan@fkip.uns.ac.id

ABSTRAK

Enzim fitase melepas molekul fosfor pada atom C dari benzena Inositol fitat. Tetapi fitase memiliki kelemahan tidak mampu bertahan terhadap kondisi ekstrim dalam lambung nonruminansia. Solusi dalam penelitian ini yaitu fitase dienkapsulasi menggunakan alginat. Penelitian ini bertujuan mengkarakterisasi fitase setelah dienkapsulasi menggunakan alginate. Hasil penelitian ini yaitu fitase yang dienkapsulasi memiliki aktivitas tertinggi pada pH 6,0, sedangkan fitase tanpa enkapsulasi pada pH 5,0. Suhu optimum untuk aktivitas tertinggi fitase yang dienkapsulasi yaitu 70ºC, sedangkan fitase tanpa enkapsulasi 37ºC. Untuk perlakuan penambahan ion logam, aktivitas tertinggi fitase yang dienkapsulasi terjadi dengan penambahan 0,1 mM Fe^{2+} dan 1,0 mM Ca^{2+}, sedangkan fitase tanpa enkapsulasi dengan penambahan 0,1 mM Fe^{2+}. Berdasarkan hasil penelitian ini, fitase yang dienkapsulasi memiliki keunggulan lebih banyak dibandingkan dengan fitase tanpa enkapsulasi, karena mampu bertahan pada pH dan suhu tinggi, dan beberapa efek ion logam.

Kata Kunci: alginat, asam fitat, enkapsulasi, fitase, fitase rekombinan

ABSTRACT

Phytase enzymes release phosphorus molecules on the C atom from benzene inositol phytate. But phytase has the disadvantage of being unable to withstand extreme conditions in the non-ruminant stomach. The solution in this research was phytase encapsulated using alginate. This study aims to characterize phytase after being encapsulated using alginate. The results of this study were the encapsulated phytase had the highest activity at pH 6.0, while the unencapsulated phytase at pH 5.0. The optimum temperature for the highest activity of the encapsulated phytase was 70ºC, while the unencapsulated phytase 37ºC. For treatment of metal ion addition, the highest activity of the encapsulated phytase occurred with the addition of 0.1 mM Fe^{2+} and 1.0 mM Ca^{2+}, while the unencapsulated phytase with the addition of 0.1 mM Fe^{2+}. Based on the results of this study, the encapsulated phytase had more advantages compared to the unencapsulated phytase, as the former withstand high pH and temperature, and some metal ion effects.

Keywords: *alginate, encapsulation, characterization, phytate, recombinant phytase*

Received: *31 July 2018* ***Accepted:*** *25 February 2020* ***Published:*** *22 July 2020*

INTRODUCTION

Phytate (*myo*-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, IP6) is an abundant plant constituent (Gerke 2015; Cowieson et al. 2017; Savita et al. 2017) which comprises of 1–5% of the total weight of nuts, cereals, oilseeds, pollen, and other legumes. Phytate is the main storage form of inositol and phosphorus (P) in the plant seed (Naves et al. 2012; Chen et al. 2015). Plant seeds contain usually 65–80% (Gull et al. 2013; Kaur et al. 2017; Shah and Desai 2017; Neira-Vielma et al. 2018) and sometimes up to 90% (McKie and McCleary 2016) of total phosphorus form of phytate. This organically bound phosphorus is unavailable for simple stomach animals because they lack significant phytase activity in their gastrointestinal tract (McKie and McCleary 2016; Megazyme 2017). Besides, phytate tends to reduce the bioavailability of multivalent cations such as Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, as well as amino acids (Sajidan et al. 2015). To provide the animals with sufficient phosphorus, their feeds are generally supplemented with inorganic phosphate. However, this tends to increase phosphate content in animal wastes (Mupondi et al. 2018) leading to water pollution (Godoi et al. 2017). This has become a great concern especially in areas of intensive livestock management, and thus supplementation of animal feed with phytase enzyme was identified as a proper solution.

Feed supplementation with phytase (McKie and McCleary 2016) was shown to result in significantly increased P digestibility in monogastric animals and hence generally tended to increase P retention by 40% (Fei et al. 2013; Mogal et al. 2017). The effect of phytase on the bioavailability of P has also been evaluated in some fish species either by pretreatment of feed with phytases or direct addition of phytase to fish feed.

Phytase can be grouped according to the C-atom on the inositol ring where phytate dephosphorylation is initiated. 3-phytase (EC 3.1.3.8), mainly identified in microorganisms, starts phytate dephosphorylation at position D3; 6-phytase (EC 3.1.8.26), mainly present in plants, at position D6 or L6; and 5-phytase (EC 3.1.3.72), identified in legumes and a rumen bacteria *Selenomonas* at position D5 (Suleimanova et al. 2015).

Based on the active site, phytase can be classified as cysteine phytases or protein tyrosine phytases (Ma et al. 2012; Irshad et al. 2017), histidine acid phytases (Ali et al. 2017), purple acid phytases, or beta-propeller phytases (Sajidan et al. 2004; Chen et al. 2015). So far only histidine acid phytases have found application as feed supplements.

A major challenge in the industrial application of phytase was identified to be its intrinsic stability. As a protein, phytase easily denatures and thus various process conditions such as temperature or pH can result in a decrease or complete loss in enzymatic activity (Homaei et al. 2013). Therefore different approaches to stabilize phytase enzymes have been applied.

One of these approaches is encapsulation. Encapsulation was shown to be capable of stabilizing enzymes without changing their character. Encapsulated enzymes were shown to work efficiently and the material used for encapsulation must be non-toxic. Thus, no negative effect on humans, animals, and the environment is expected. Compared to the free enzymes in solution, encapsulated enzymes were reported to be less prone to changes such as pH or temperature in the environment (Tsai and Meyer 2014; Zdarta et al. 2017). Furthermore, the heterogeneous encapsulated enzyme system enables easy recovery of enzymes and reaction products (Fernandez-Lafuente 2017), the reuse of the encapsulated enzymes, continuous enzymatic process operations, and rapid cessation of the enzymatic reaction (Homaei et al. 2013; Zdarta et al. 2017).

Alginate has been reported to be a suitable material for enzyme encapsulation (Ali et al. 2017). It is safe to use and can be obtained at a fairly affordable cost. In addition, substrates and reaction products trapped in the alginate matrix can spread easily (Tsai and Meyer 2014; Ali et al. 2017). This study aims to characterize recombinant phytase (phyK) from *Klebsiella* sp. ASR1 after encapsulated using alginate, to see the differences in activity characteristics through the effects of pH, temperature, and metal ions.

MATERIALS AND METHODS

Location and time of research

This research was conducted from May 2017 to March 2018, in the 4 Biology Laboratory, Faculty of Mathematics and Natural Sciences, and the central Chemistry Laboratory, Sebelas Maret University, Surakarta, Central Java.

Materials

The materials used in this study were alginate, phytate (Sigma), tryptone (Fluka BioChemika), yeast extract (Oxoid), sodium chloride (Merk), agar (Pronadia), ampicillin (Kalbe Farma) IPTG (Promega), potassium dihydrogen phosphate (Phyedu media), acetic acid (Phyedu media), ammonium molybdate, ammonium metavanadate (Sigma Aldrich), imidazole (Sigma Aldrich) and Ni-NTA agarose (Qiagen).

Strains, production, and purification

Escherichia coli BL21 (DE3) transformed by Nuhriawangsa et al. (2008) with pET22b (+)–Plasmid-Vector product (Novagen 69744-3) harbouring the PhyK gene from *Klebsiella* sp. ASR1 (Sajidan et al. 2004) was used in the study. The recombinant *E. coli* BL21 (DE3) phyK was then rejuvenated in LB (consisting of 1 g NaCl, 2 g tryptone, 1 g yeast extract, and 2 g agar; all dissolved in 100 mL distilled water + ampicillin) solid medium supplemented with 100 µg mL^{-1} ampicillin. The recombinant phytase enzyme was produced in LB liquid medium (consisting of 1 g NaCl, 2 g tryptone, and 1 g yeast extract; all dissolved in 100 mL distilled water 100 mL + 100 µg mL^{-1} ampicillin and 1.5 mM IPTG), also supplemented with ampicillin. The culture was incubated in a shaking incubator at 100 rpm at room temperature for 6 hours (1.5 mM IPTG was added at 2 hours after the culture was stored in the shaking incubator). Thereafter, the bacterial cells were collected by centrifugation at 3,500*g* for 5 minutes. The supernatant obtained was used as a source of crude extracellular phytase, which was then purified using Ni-NTA agarose as described by Sajidan et al. (2004, 2015). The supernatant was added to the chromatographic column containing Ni-NTA agarose along with a pH 5.0 buffer and a 200 mM imidazole solution. The enzyme that dripped out of the affinity column was collected into an ice-cold container.

Standard phytase assay

Standard phytase activity assay was performed as described by Sajidan (2002). The reaction mixture consisted of 150 µL enzyme solution and 750 µL substrate solution (0.4% Na-phytate in 0.82% Na acetate buffer, pH 5). The reaction mixture was incubated at 37ºC for 1 hour. The enzymatic reactions were stopped by addition of 2400 µL stop solution containing ammonium molybdate solution (2.352 g ammonium molybdate, 2 mL HNO_3, 100 mL distilled water) : HNO_3 water : 10% ammonium metavanadate solution in the ratio 1.5 : 1 : 2 : 1.5 (v/v). Absorbance was measured using a UV-VIS spectrophotometer at 415 nm and a calibration curve was prepared using a 0,03834 mg mL^{-1} KH_2PO_4 standard solution series (0.025, 0.05, 0.075, 1, 2, 3, and 4 mL) (Widowati et al. 2001, Wulandari 2011). The standard solutions were each added with 6,25 mL of the molybdate-vanadate solution, incubated for 10 minutes, and diluted up to 25 mL before being measured spectrophotometrically at λ 415 nm. The absorbance data obtained were then converted into enzyme activity unit using the formula below:

Activity of phytase (U/mL) =

$$\frac{[(ODx \times OD) \times 1000 \times Df]}{T \times molecular\ weight\ of\ phytase}$$

where

ODx = Absorbance assay samples
OD = Control absorbance
1000 = Factor to convert
Df = Dilution factor
T = Incubation time

Molecular weight of phytase = 47.3 kDa

Encapsulation of phytase

Alginate was dissolved in 9.26 mL phosphate buffer pH 5.0 by heating to 100ºC. After cooling down to 45ºC, 2 mL of the purified enzyme solution was added. The phytase alginate mix was put into an ice bath. After hardening, the alginate containing the trapped phytase enzyme was cut into 0.75 mm pieces as described by Oktavia et al.

Tabel 1. Phytase preparation and activity

No	Preparation	Activity (U mL^{-1})
1	Crude phytase	0,090
2	Ni-NTA-purified phytase	0,150
3	Encapsulated phytase	0,2856

Note: Molecular weight of phytase is 47,3 kD (Sajidan et al. 2004)

(2013) and Saxena et al. (2017). Exactly 200 mg of the alginate pieces were washed with double distilled water. Enzyme activity was determined using the standard phytase assay and the remaining alginate pieces were stored at 4ºC until further use (Saxena et al. 2017).

pH, temperature, and metal ions

For pH optimum determination, phytase activity assay was performed using the following buffers: pH 3.0: 0.1 M citric acid-sodium citrate, pH 4.0–5.0: 0.2 M sodium acetate-acetic acid, pH 5.0–8.0: 0.2 M sodium phosphate, and pH 9.0: 0.1 M glycine-NaOH at 37ºC. The temperature profile was determined using the standard phytase assay at 37, 40, 50, 60, 70, 80, and 90ºC. The effect of metal ions on phytase activity was determined using the standard phytase assay in the presence of 0.1 M and 1.0 M Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}.

RESULTS AND DISCUSSION

Culture, encapsulation, and phytase assay

Figure 1A shows a white round shape with a slick surface of the recombinant *E. coli* containing PhyK gene, while the liquid medium with high turbidity indicates bacterial growth. The activity of crude (supernatant) enzyme was determined and found to be 0,090 U mL^{-1}, and the activity after purification using Ni-NTA 0.150 U mL^{-1} (Figure 1B and Table 1). The encapsulated phytase is as shown in Figure 1C and its activity was 0.2856 U mL^{-1} (Table 1), which was higher than the activity of unencapsulated phytase (Figure 2).

Effect of pH on the phytase activity

The results of the phytase assay in this study showed similarities with that of Niu et al. (2017). In this study the activity of

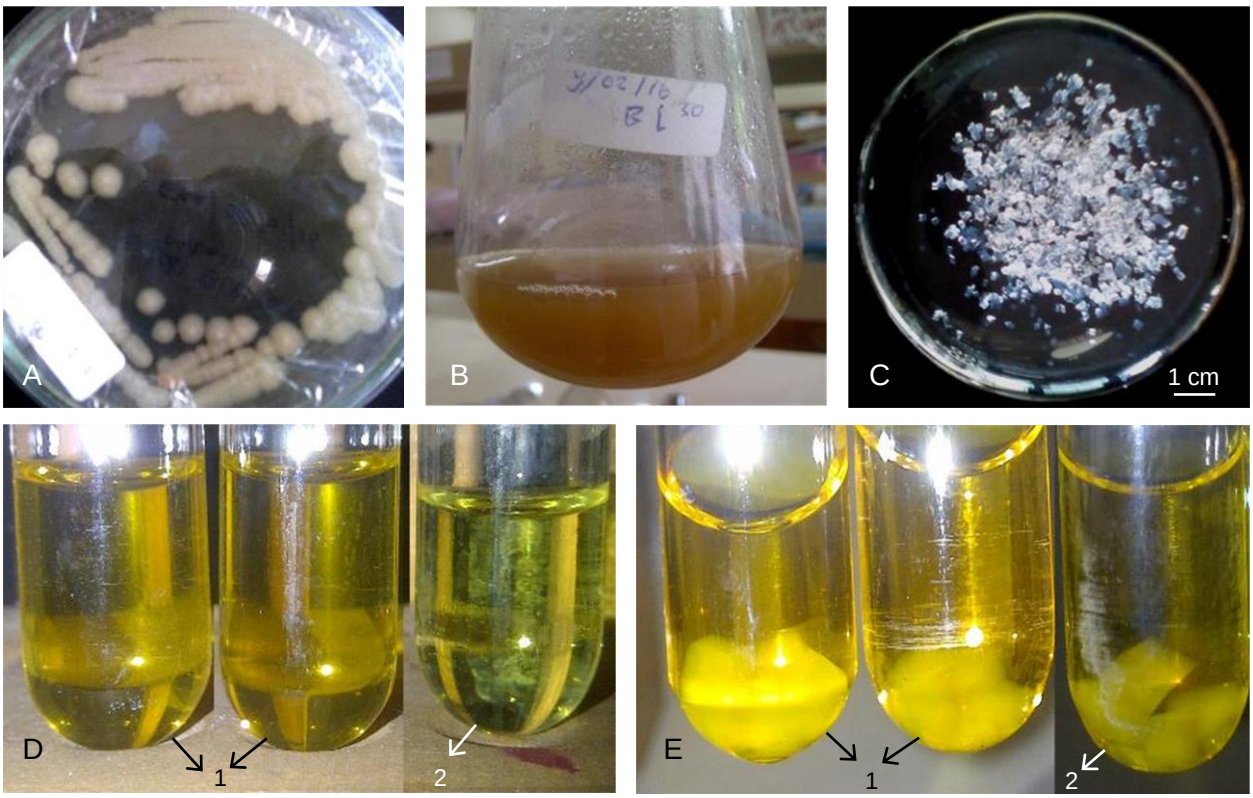

Figure 1. Bacterial culture of *E. coli* containing phyK gene on Lauria Bertani solid medium added with ampicillin (A) and in Lauria Bertani liquid medium supplemented with ampicillin and phytate (B); alginate-encapsulated phytase (C); solution mixture containing unencapsulated phytase (D) and alginate-encapsulated phytase (E), respectively, with phytate (1) and without phytate as control (2)

unencapsulated phytase was observed to have the highest activity at pH 5.0. Their activity was also observed to decrease significantly at pH 6.0, 7.0, 8.0 and 9.0. The alginate-encapsulated phytase exhibited the highest activity at pH 6.0 (Figure 2). The difference in the activity was due to encapsulation of recombinant phytase enzyme using alginate. It was also caused by the fact that the *E. coli* used in the cloning of the phyK gene from *Klebsiella* sp. ASR1, which was from Indonesia rice soil study by Sajidan et al. (2004), in which the phytase had a single optimum pH of 5.0. The phytase activity decreased at pH above 7.0.

Some studies suggest that immobilization or encapsulation techniques tend to alter the characteristics of enzymes or cells in terms of optimum pH (Cardoso et al. 2019, Reis et al. 2019). In this study, the encapsulation of phytase enzymes using alginate showed the highest activity at pH

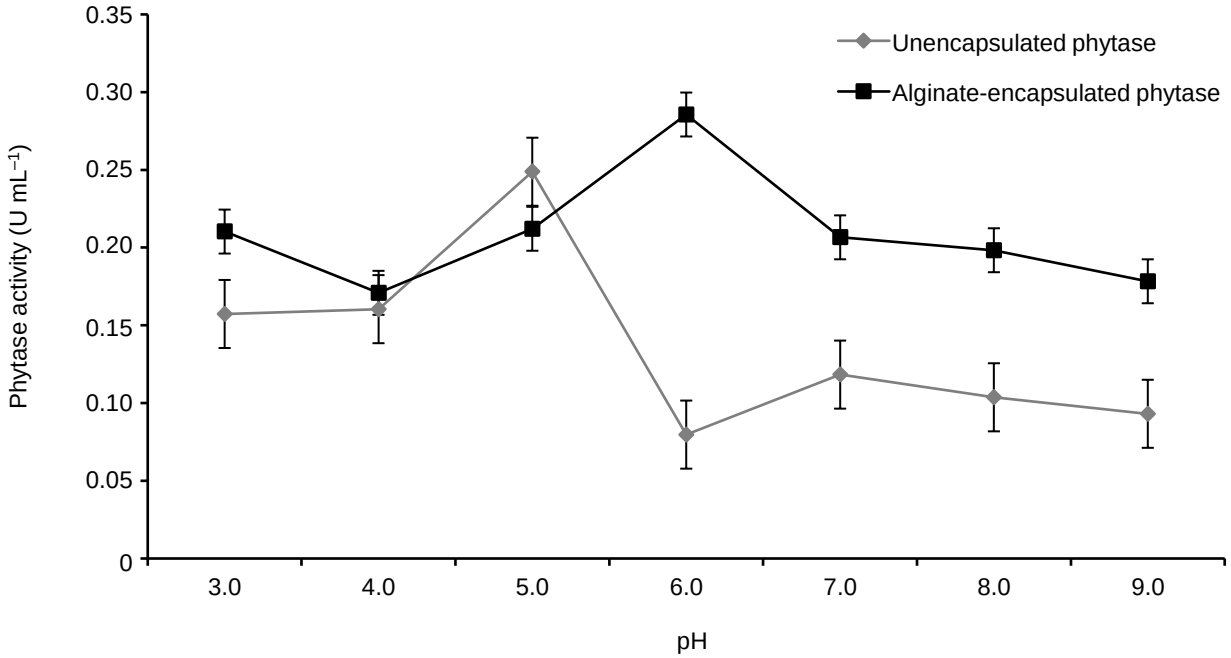

Figure 2. Activity of phytase at different pHs

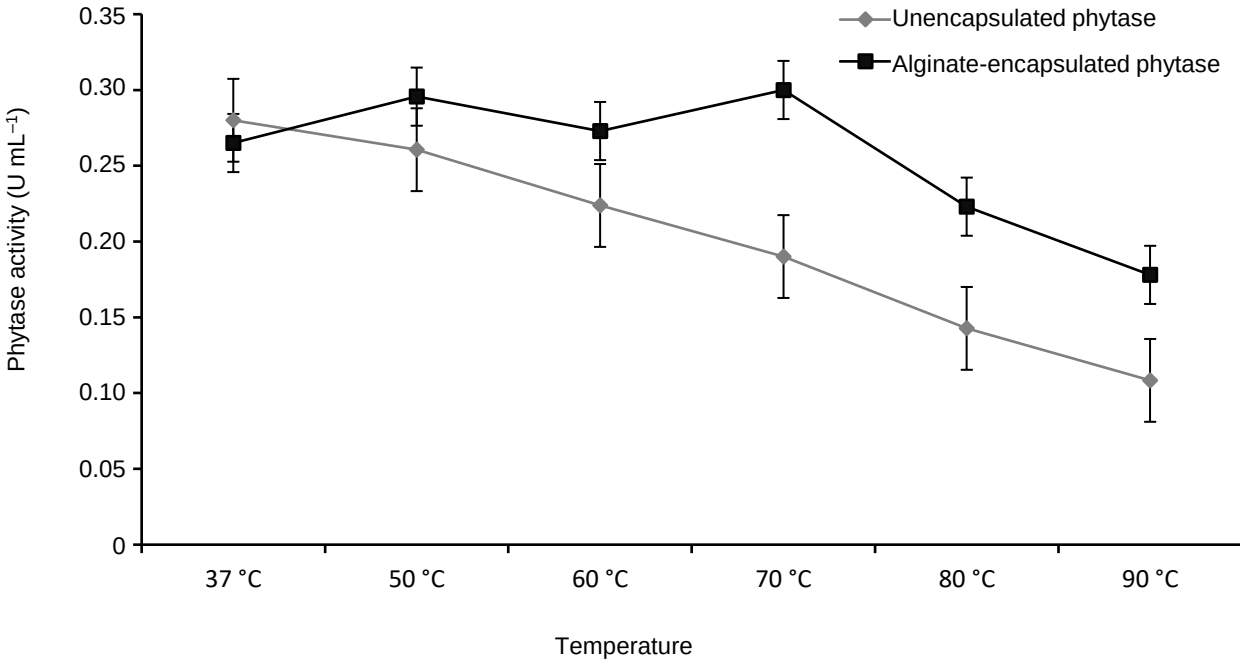

Figure 3. Activity of phytase at different temperatures

6.0. This is because the release of the enzyme from the alginate nucleus corresponds to a low pH/acid pH solution (Kyzioł et al. 2017). In addition to that, Zhang et al. (2016) described that alginate-derived proteins tend to be efficient against low pH 3.0, 5.0 and 7.0. This attribute is caused by the presence of strong electrostatic attraction between cationic proteins and anionic alginate molecules consequently the protein molecules in alginate become stronger and more resistant. Then as the pH increases its activity decreases and this is again linked to the weakening of electrostatic forces between the molecules of the dehydrated proteins and alginate at an increasing pH. Saxena et al. (2017) also explained that the activity of alginate-encapsulated enzymes will increase as the concentration of alginate increases to 3%.

Effect of temperature

Temperature may affect the activity and stability of encapsulated enzymes. Results from this study showed that the highest activity of the unencapsulated phytase occurred at 37ºC, but in general the activity decreased as the temperature was increased. The unencapsulated phytase activity was observed to decrease significantly as the temperature was increased from 60ºC to 90ºC. On the other hand, the alginate-encapsulated phytase in general showed increasing activity from 37ºC to 70ºC, but decreased significantly at 80–90ºC (Figure 3). Sajidan et al. (2004) reported unencapsulated phytase with an optimal

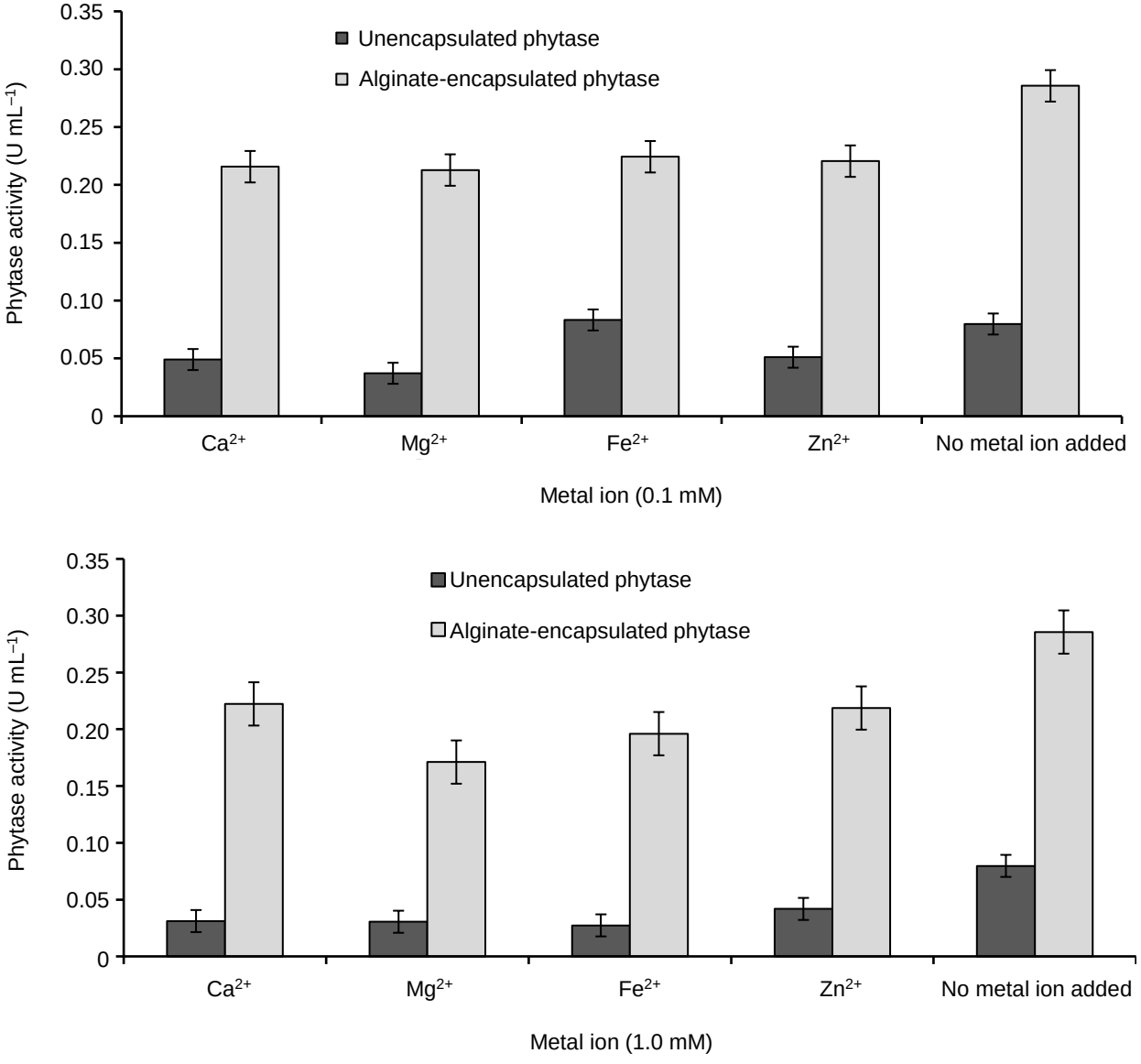

Figure 4. Activity of phytase in the presence of metal ions at two different concentrations

activity at the temperature of 45°C. The activity tended to decrease at temperature above 50ºC, and at 45–50ºC for phytase from *Klebsiella pneumonia*.

Other researchers reported the activity of the original *E. coli* phytase which was higher at the temperature between 55º and 60ºC (Naves et al. 2012). This activity difference with encapsulated phytase is likely to be caused by encapsulations which enhance the phytase enzyme to withstand extreme temperatures.

Generally, high temperature increases the activity rate of encapsulated phytase. This is because, at high temperatures, molecules move faster, so enzymes tend to come into contact with the substrate very quickly (Rezakhani et al. 2014). Free glycosidase enzyme is a good example (Keerti et al. 2014). The author reported the enzyme's optimal temperature to be 60ºC. However, after encapsulation using alginate, the optimal temperature increased up to 80ºC. This is because secondary and hydrophobic interactions of immobilized or encapsulated enzymes may interfere with conformational flexibility requiring higher temperatures for enzyme molecules to recognize and achieve appropriate conformations for their reactivity to remain (Rezakhani et al. 2014).

Effect of metal ions

In this study, the presence of the metal ions added decreased the activity of both encapsulated and unencapsulated phytases. The activity of alginate-encapsulated phytase was lower in the presence of the metal ions than that without metal ions addition, regardless of the metal ions and the concentration added, and so was the unencapsulated enzyme. But in all of the treatment, the activity of the encapsulated phytase was still far above that of its unencapsulated counterpart (Figure 4). For the treatment of metal ion addition, the highest activity of the encapsulated phytase occurred with the addition of 0.1 mM Fe^{2+} and 1.0 mM Ca^{2+}, while the unencapsulated phytase with the addition of 0.1 mM Fe^{2+}.

Enzymes have properties and characteristics that are regulated by ions or molecules. The specific properties of some of these ions can be activators and inhibitors. Suleimanova et al. (2015) described that ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} can increase enzymatic activity more than 2-fold. This mechanism occurs because cofactors in the form of metal ions (auxiliary molecules) can change the shape of the active side of the enzyme and act as a stabilizer. Study by Yao et al. (2014) on different microbial phytases suggested that unencapsulated phytase from *E. coli* phy (ycE) had an activity which was more stable in the presence of Ca^{2+} while study by Wang et al. (2015) described that the activity of *E. coli* DH5α phytase was not affected by the significant decrease of Fe^{2+} ions.

CONCLUSION

Encapsulation using alginate of the recombinant phytase (phyK) from *Klebsiella* sp. ASR1 resulted in the enzyme highest activity at low pH. The enzyme maintained its activity at higher temperature. Besides, the effect of metal ions also shows higher activity. The results showed that the characterization of alginate-encapsulated enzyme based on the variation of pH, temperature and metal ions tended to increase the enzyme activity compared to the unencapsulated one. The use of alginate for phytase encapsulation was quite effective and the results obtained were very promising to be further developed and applied.

REFERENCES

Ali S, Zafar W, Shafiq S, Manzoor M (2017) Enzymes immobilization: An overview of techniques, support materials and its applications. Int J Sci Technol Res 6:64–72

Chen C, Cheng K, Ko T, Guo R (2015) Current progress in phytase research: Three-dimensional structure and protein engineering. ChemBioEng Rev 2:76–86. doi: 10.1002/cben.201400026

Cowieson AJ, Ruckebusch JP, Sorbara JOB, Wilson JW, Guggenbuhl P, Tanadini L, Roos FF (2017) A systematic view on the effect of microbial phytase on ileal amino acid digestibility in pigs. Anim Feed Sci Technol 231:138–149. doi: 10.1016/j.anifeedsci.2017.07.007

Fei B, Xu H, Zhang F, Li X, Ma S, Cao Y, Xie J, Qiao D, Cao Y (2013) Relationship between *Escherichia coli* AppA phytase's thermostability and salt bridges. J Biosci Bioeng 115:623–627.

doi: 10.1016/j.jbiosc.2012.12.010
Fernandez-Lafuente R (2017) Special issue: Enzyme immobilization 2016. Molecules 22:E601. doi: 10.3390/molecules22040601
Gerke J (2015) Phytate (inositol hexakisphosphate) in soil and phosphate acquisition from inositol phosphates by higher plants. A Review. Plants 4:253–266. doi: 10.3390/plants4020253
Godoi RR, Andrade SFV, Machado JM, de Souza TPP, Gonçalves AFA, Lopes TB, Parachin NS, Naves LP, Lima WJN, Pereira VV, Galdino AS (2017) Fungal phytase production in different hosts: A brief review. J Microbiol Biotechnol 2:000120. doi: 10.23880/oajmb-16000120
Gull I, Hameed A, Aslam MS, Athar MA (2013) Optimization of phytase production in solid state fermentation by different fungi. Afr J Microbiol Res 7:5207–5212. doi: 10.5897/AJMR2013.6136
Homaei AA, Sariri R, Vianello F, Stevanato R (2013) Enzyme immobilization: An update. J Chem Biol 6:185–205. doi: 10.1007/s12154-013-0102-9
Irshad M, Asgher M, Bhatti KH, Zafar M, Anwar Z (2017) Anticancer and nutraceutical potentialities of phytase/phytate. Int J Pharmacol 13:808–817. doi: 10.3923/ijp.2017.808.817
Kaur R, Saxena A, Sangwan P, Yadav AN, Kumar V, Dhaliwal HC (2017) Production and characterization of a neutral phytase of *Penicillium oxalicum* EUFR-3 isolated from Himalayan region. Nusantara Biosci 9:68–76. doi: 10.13057/nusbiosci/n090112
Keerti, Gupta A, Kumar V, Dubey A, Verma AK (2014) Kinetic characterization and effect of immobilized thermostable β-glucosidase in alginate gel beads on sugarcane juice. ISRN Biochem 2014:178498. doi: 10.1155/2014/178498
Kyzioł A, Mazgała A, Michna J, Regiel-Futyra A, Sebastian V (2017) Preparation and characterization of alginate/chitosan formulations for ciprofloxacin-controlled delivery. J Biomater Appl 32:162–174. doi: 10.1177/0885328217714352
Ma X-F, Tudor S, Butler T, Ge Y, Xi Y, Bouton J, Harrison M, Wang Z-Y (2012) Transgenic expression of phytase and acid phosphatase genes in alfalfa (*Medicago sativa*) leads to improved phosphate uptake in natural soils. Mol Breeding 30:377–391. doi: 10.1007/s11032-011-9628-0
McKie VA, McCleary BV (2016) A novel and rapid colorimetric method for measuring total phosphorus and phytic acid in foods and animal feeds. J AOAC Int 99:738–743. doi: 10.5740/jaoacint.16-0029
Megazyme (2017) Phytic acid (Phytate)/Total phosphorus – Measured as phosphorus released by phytase and alkaline phosphatase. Assay procedure. Megazyme. Wicklow, Ireland
Mogal C, Singh D, Mehta A, Ahmad T, Suthar K (2017) Isolation and biochemical characterization of phytase from different sources. J Appl Biotechnol Bioeng 2:152–155. doi: 10.15406/jabb.2017.02.00039
Naves L de P, Corrêa AD, Bertechini AG, Gomide EM, Santos CD dos (2012) Effect of pH and temperature on the activity of phytase products used in broiler nutrition. Rev Bras Ciênc Avíc 14:181–185. doi: 10.1590/S1516-635X2012000300004
Neira-Vielma AA, Aguilar CN, Ilyina A, Contreras-Esquivel JC, Carneiro-da-Cunha MG, Michelena-Alvarez G, Martínez-Hernández JL (2018) Optimized production of phytase by solid-state fermentation using triticale as substrate and source of inducer. Afr J Biotechnol 17:81–90. doi: 10.5897/AJB2017.16267
Cardoso FSN, Koblitz MGB, Ortiz GMD, de Carvalho JLV, de Carvalho LMJ (2019) Study of the parameters used in the encapsulation of commercial pectinase in calcium alginate and its effect on its catalytic activity. Food Sci Technol Campinas 39:247–252. doi: 10.1590/fst.31518
Niu C, Yang P, Luo H, Huang H, Wang Y, Yao B (2017) Engineering the residual side chains of HAP phytases to improve their pepsin resistance and catalytic efficiency. Sci Rep 7:42133. doi: 10.1038/srep42133
Nuhriawangsa AMP, Bachruddin Z, Sajidan, Wibowo A (2008) Karakterisasi fitase kasar (ekstraseluler) dari bakteri rekombinan *E. coli* BL21 (DE3)+pEAS1. Media Kedokt Hewan 24:124–131
Oktavia R, Suharti, Susanti E (2013) Karakterisasi enzim bromelin yang diamobilisasi dalam agar komersial. J

Online Kimia Universitas Negeri Malang 2:1–10

Mupondi LT, Mnkeni PNS, Muchaonyerwa P, Mupambwa A (2018) Vermicomposting manure-paper mixture with igneous rock phosphate enhances biodegradation, phosphorus bioavailability and reduces heavy metal concentrations. Heliyon 4:e00749. doi: 10.1016/j.heliyon.2018.e00749

Reis CLB, de Sousa EYA, de Franca Serpa J, Oliveira RC, dos Santos JCS (2019) Design of immobilized enzyme biocatalysts: Drawbacks and opportunities. Quim Nova 42:768–783. doi: 10.21577/0100-4042.20170381

Rezakhani N, Molaei A, Parivar K, Khayati M, Etemadzade S (2014) Immobilization of protease in biopolymers (mixture of alginate-chitosan). J Paramed Sci 5:108–113. doi: 10.22037/jps.v5i4.7858

Sajidan (2002) Molekulare Charakterisierung einer Phytase (Myo-Inositol Hexakisphosphate Hydrolase) und von Phosphatasen aus Bakterienisolaten indonesischer Reisfelder (*Klebsiella pneumoniae*). Dissertation. Institut für Biologie, Humboldt-Universität zu Berlin

Sajidan A, Farouk A, Greiner R, Jungblut P, Müller E-C, Borriss R (2004) Molecular and physiological characterisation of a 3-phytase from soil bacterium *Klebsiella* sp. ASR1. Appl Microbiol Biotechnol 65:110–118. doi: 10.1007/s00253-003-1530-1

Sajidan, Wulandari R, Sari EN, Ratriyanto A, Weldekiros H, Greiner R (2015) Phytase-producing bacteria from extreme regions in Indonesia. Braz Arch Biol Technol 58:711–717. doi: 10.1590/S1516-89132015050173

Savita PD, Suvarna VC, Annu T, Balakrishna AN, Kanchanashri B, Yallappa M (2017) Characterization and identification of phytate solubilizing yeasts isolated from food grains. Int J Curr Microbiol Appl Sci 6:1184–1192. doi: 10.20546/ijcmas.2017.604.145

Saxena R, Kumar M, Tomar RS (2017) Nanobiotechnology: A new paradigm for crop production and sustainable agriculture. Res J Pharm Biol Chem Sci 8:823–832

Shah K, Desai P (2017) Isolation and characterization of phytase enzyme from *Escherichia coli*. Int J Pharm Bio Sci 8:474–478. doi: 10.22376/ijpbs.2017.8.2.b474-478

Suleimanova AD, Beinhauer A, Valeeva LR, Chastukhina IB, Balaban NP, Shakirov EV, Greiner R, Sharipova MR (2015) Novel glucose-1-phosphatase with high phytase activity and unusual metal ion activation from soil bacterium *Pantoea* sp. strain 3.5.1. Appl Environ Microbiol 81:6790–6799. doi: 10.1128/AEM.01384-15

Tsai CT, Meyer AS (2014) Enzymatic cellulose hydrolysis: Enzyme reusability and visualization of β-glucosidase immobilized in calcium alginate. Molecules 19:19390–19406. doi: 10.3390/molecules191219390

Wang T, Hao Y, Li W, Liu Y (2015) Enzymatic properties of phytase from *Escherichia Coli* DH5α. Proceedings of the International Conference on Chemical, Material and Food Engineering:88–92. doi: 10.2991/cmfe-15.2015.22

Widowati S, Andriani D, Riyanti EI, Raharto P, Sukarno L (2001) Karakterisasi fitase dari *Bacillus coagulans*. Dalam: Mariska I, Somantri IH, Sutrisno, Machmud M, Simanungkalit RDM, Suyono, Orbani IN (eds) Prosiding Seminar Hasil Penelitian Rintisan dan Bioteknologi Tanaman, 30-31 Januari 2001. BPBTP, Bogor, hal 245–255

Wulandari R (2011) Analisis gen 16S rRNA pada bakteri penghasil enzim fitase. Tesis, Universitas Sebelas Maret Surakarta

Yao M, Lu W, Chen T, Wang W, Fu Y, Yang B, Liang A (2014) Effect of metals ions on thermostable alkaline phytase from *Bacillus subtilis* YCJS isolated from soybean rhizosphere soil. Ann Microbiol 64:1123–1131. doi: 10.1007/s13213-013-0751-5

Zdarta J, Jedrzak A, Klapiszewski Ł, Jesionowski T (2017) Immobilization of cellulase on a functional inorganic-organic hybrid support: Stability and kinetic study. Catalysts 7:374. doi: 10.3390/catal7120374

Zhang Z, Zhang R, Zou L, McClements DJ (2016) Protein encapsulation in alginate hydrogel beads: Effect of pH on microgel stability, protein retention and protein release. Food Hydrocolloids 58:308–315. doi: 10.1016/j.foodhyd.2016.03.01