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EXTRACELLULAR ALKALINE PHOSPHATASE FROM MANGROVE SOIL YEAST

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ABSTRACT

Alkaline phosphatase is a widely used enzyme in research and industry. Yeast is one of a microbial group that has the potency to produce alkaline phosphatase. This study aims to screen yeast from mangrove in Surabaya's East Coast that produce f alkaline phosphatase and to optimize the production of these enzymes.Screening test includes the measurement of phosphate solubilization index and concentration of soluble phosphate. Yeast with potential of phosphate solubilization will be used to measured the enzyme activity and also further optimizedfor extracellular alkaline phosphatase production.Optimization was done by determining the incubation time and a variety of combinations of phosphate source and temperature. The incubation time was adjustedto 7 days. $Ca_3(PO_4)_2$ *and* KH_2PO_4 *as the phosphate sourceand incubation temperature were adjusted to 29° C, 45° C, and 55° C. The results showed that isolates with the highest phosphate solubilization index were W1.1 and G3.2 (1.18 for W1.1 and1.15 for G3.2). Yeast with the ability to solubilize phosphate was assumed to be Candidagenus.W1.1 andG3.2 produced soluble phosphate in a concentration of 0.50 ppm and 0.77 ppm at the 7th day respectively. Candida G3.2 has higher enzyme activity than Candida W1.1. Candida G3.2 has optimal production for 3 days incubation time with a combination of temperature 29 °C and* $Ca_3(PO4)_2$ *as the phosphate source which caused 97.8 U/mL enzyme activity.*

Keywords: alkaline phosphatase, enzyme activity, mangrove soil yeast

I. INTRODUCTION

Yeast is widely used in industrial processes, such as the production of alcoholic beverages, food, fodder and synthesis of various metabolic products. The enzyme is one of the metabolic products that are often explored in the industry (Norouzian, 2008). Based on the adaptability of yeast in extreme environments, yeast from the marine area including mangrove area also has potential over other terrestrial yeasts, related with salt tolerance, enzyme production, and the production of ethanol and other fermentation processes (Kandasamy et al., 2012).

Yeast isolated from the marine area can produce extracellular hydrolytic enzymes (Chi et al., 2009). One of the hydrolytic enzymes is alkaline phosphatase. Alkaline phosphatase (orthophosphate monoester phosphohydrolases, EC 3.1.3.1) is metalloenzyme (Sasajima et al., 2010) which is responsible for breaking the phosphate group (dephosphorylation) of different types of molecules, including nucleotides, proteins, and alkaloids (Holander, 1971). As the name implies, alkaline phosphatase has optimal catalytic activity at alkaline pH (Rin et al., 2000). In various organisms, alkaline phosphatase has an important role in the process of transportation and phosphate metabolism (Fernandes et al., 2008).

Alkaline phosphatase is widely used in the fields of clinical diagnostics,and molecular biology as a quantitative measurement of biochemical markers in diseases, such as ELISA (Guo et al., 2018) () The importance of alkaline phosphatase in the field of medical science and molecular biology made alkaline phosphatase widely explored in the study of scientific and commercial interest (Muginova et al., 2007). Commercial alkaline phosphatase nowadays is extracted from the intestines of cattle and *Escherichia coli* (Sigma-Aldrich, 2013). Because of the expensive process of extracting enzymes from animals, publicperception in potential dangers of *E. coli* strains, and low adaptability of bacteria compared to yeast (Lambertz et al., 2014),yeast can be used as an alternative source for the production of alkaline phosphatase.

Alami and Shovitri, (2015 has been obtained yeast from Surabaya East Coast region. However, those isolates have not been tested its potential in the production of alkaline phosphatase. Therefore, this study aimed to screen yeast from mangrove in Surabaya's East Coast that potential to produce alkaline phosphatase and to optimize the production of these enzymes.

II. MATERIALS AND METHODS

a. Subcultures of Yeast Isolates

Yeast isolates collection of Microbiology and Biotechnology Laboratory, Biology Department,ITS, was subcultured on a slant YMEA (Yeast Malt Extract Agar) medium, and then was incubated for four days at room temperature.

b. Measurement of Phosphate SolubilizationIndex

Isolates were aseptically inoculated into a Petri dish containing Pikovskaya medium by spot inoculation method. The cultures were incubated at room temperature and observed the formation of a clear zone every 24 hoursfor 7 days. Yeast that potential in phosphate solubilization was indicated by clear zone formation around the colony (Kanti, 2006). Clear zone and the diameter of colonies counted every 24 hours for 7 days to obtain Phosphate Solubilization Index. To calculate SI (Solubilization Index) according to (Jena and Rath, 2013) used the following formula [1]where *SI* is solubilization index, x_1 is clear zone (cm), x_2 is the diameter of colonies (cm).

$$SI = \frac{X1+X2}{X2} \quad [1]$$

Two isolates with the best results from this test will be continued to the next step.

c. Measurement of Soluble Phosphate Concentration

Phosphate solubilization test is conducted to determine the strength of the isolates in dissolving phosphate, 2 isolates with the best result from the previous test were used in this test. Yeast suspension is done by taking some ose of yeast from YMEA slant to put in 100 mL of sterile physiological water to obtain 0.5 OD at a wavelength of 600 nm. About 6 mL of suspension was inserted into 8 pieces Erlenmayer which already contains 54 ml of liquid

Pikovskaya medium. The cultures were incubated at room temperature using a rotary shaker at 130 rpm. Phosphate levels were measured at each end of the incubation time, measurements were carried out after the culture was incubated for 0, 1, 2, 3, 4, 5, 6, and 7 days. The culture medium was harvested and inoculated on YMEA with pour plate method to obtain the number of colonies of the yeast at each time of incubation. In addition, about 20 ml of culture is taken, then filtered using Whatman Paper No. 42 and centrifuged (4,000 rpm for 15 minutes) (Saraswati et al., 2007). About 3 mL of the supernatant was put into a test tube. Then added 0.5 mL of phosphate reagent and allowed to stand for 1 minute. The absorbance was measured at λ 880 nm. Measurements of soluble phosphate were done by interpolating the absorbance values on the standard curve.

d. Quantitative Test of Alkaline Phosphatase by Yeast

2 isolates from the previous step were tested quantitatively using a liquid medium Pikovskaya (Kapri and Tewari, 2010) which has a composition (g / L): 0.500 yeast extract; glucose 10; Ca_3 (PO_4) $_2$ 5; $(NH_4)_2SO_4$ 0.5; KCl 0.2; $MgSO_4$ 0.1; MnSO4 0.0001; and $FeSO_4$ 0.0001. pH of the medium is set to 8 by the addition of Tris-HCl buffer (Jena and Rath, 2014). The suspension of each isolate was inoculated as much as 5% in the medium respectively. The cultures were incubated at room temperature with aeration using a rotary shaker at 130 rpm for 5 days. Furthermore, the culture was centrifuged 6000 rpm for 15 min (Qureshi et al., 2010). The supernatant was stored for enzyme activity analysis.

e. Preparation of Acclimatization Medium

Acclimatization medium is using 150 mL YMB (Yeast Malt Broth) with the addition of Ca_3 (PO_4) 2 5 g / L. Yeast isolates were inoculated on acclimatization medium aseptically and incubated until exponential phase (μ day) on a rotary shaker (Kathiresan et al., 2011).

f. Influence of Incubation Time

Culture fromacclimation medium was inoculated as much as 5% into Pikovskaya liquid medium. The

cultures were incubated at room temperature using a rotary shaker at 130 rpm for 7 days. At each day, cultures were centrifuged (6000 rpm for 15 min) (Qureshi et al., 2010). Test of enzyme activity was measured every day to determine the best incubation time. Best incubation time results will be used for next optimization stages.

g. Influence of Combination of Phosphate Source and Temperature

The test aimed to detect the appropriate substrates and temperature for the production of alkaline phosphatase.About 5 g / L of $Ca_3(PO_4)_2$ and KH_2PO_4 asphosphatesourcewere added to Pikovskaya medium with incubation temperature were 29° C, 45° C and 55° C. About 5% culture from acclimation medium were inoculated into Pikovskaya liquid medium. The cultures were incubated at different temperature using incubator shaker 130 rpm with optimum incubation time obtained from the previous stage. Cultures were centrifuged (6000 rpm for 15 min at 4 ° C) (Qureshi et al., 2010). The supernatant was stored for analysis enzyme activity.

h. Alkaline Phosphatase Activity Test

Measurement of the enzyme activity is done by mixing 1 mL substrate solution (2 mg / mL p-NPP in 1 M Tris-HCl buffer solution at pH 10.0) and 1 ml of crude enzyme extract (supernatant) and incubated at 30 ° C for 10 minutes. The reaction was stopped by adding 0.05 ml 4 M NaOH (Qureshi et al., 2010). Absorbance was measured using a UV-Vis spectrophotometer with λ 410 nm (Jena and Rath, 2014). Control is 1 mL substrate and 1 mL of medium without inoculated yeasts with the same treatment. Activities per unit (U) are indicated in mol p-nitrophenol per minute (Jena and Rath, 2014). Measurement of the enzyme activity is done by interpolating the absorbance values on the p-nitrophenol standard curve then calculated by the following formula [2] where *UA* is enzyme activity (unit/mL), *x* is p-nitrophenol concentration (µL), *v* is volume of total reaction (mL), *t* is reaction time (minute), *s* is enzyme volume (mL) :

$$UA = \frac{x.V}{t.s} \quad [2]$$

III. RESULTS AND DISCUSSION

The measurement of phosphat solubilization index showed that Solubilization Index (SI) of isolates were varied, there are 2 isolates that have higher SI when compared to other isolates (Figure 1). W1.1 isolate has SI value of 1.18, and G3.2 isolate has SI value of 1.15. The ability of yeast isolates in phosphate solubilizationis indicated from the formation of clear zone on Pikovskaya solid medium. Pikovskaya Medium contain calcium phosphate ($Ca_3(PO_4)_2$) as P source (Raharjo et al., 2007). Phosphate solubilization by microorganisms can be done by both chemically and biologically. The mechanism of chemically dissolving phosphate is a major phosphate solubilization mechanisms done by microorganisms. The microorganisms excrete a number of low molecular weight organic acids like oxalic, succinic, tartaric, citric, etc. (Ginting et al., 2006). Biological phosphate solubilization occurs because the microorganisms produce enzymes include phosphatase enzyme (Ginting et al., 2006). Two Isolates with the highest SI values will be further identified and tested the levels of soluble phosphate.

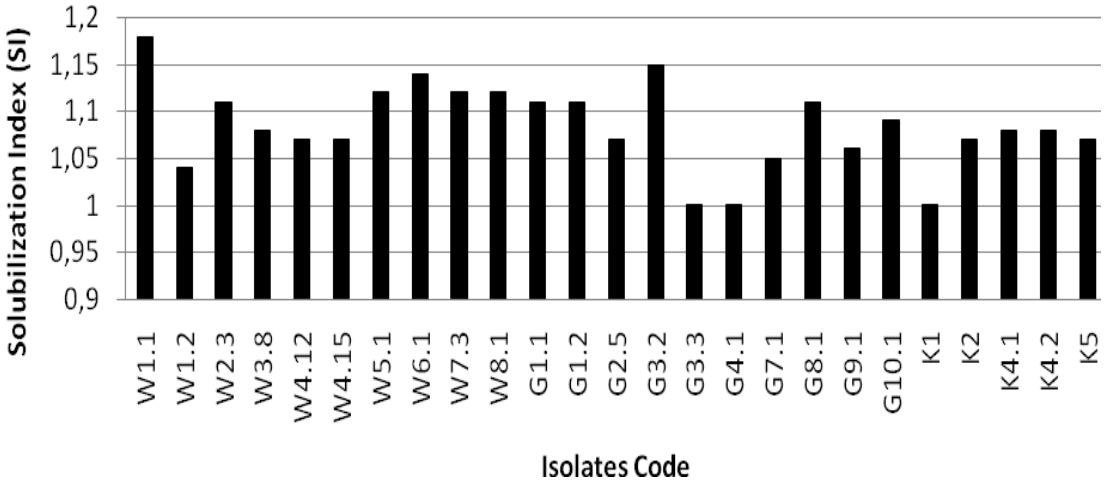

Figure.1. Solubilization Index (SI) of yeast isolates

Yeast identificationis based on the"The yeasts a Taxonomic Study" (Kurtzman and Fell, 1998). The stages include macroscopic and microscopic observation, physiological or biochemical test, ascospores test and

growth test in a liquid medium. The test results showed that W1.1and G3.2 isolates have similarities with the character of *Candida*. Some characters that strengthen the assumption that these isolates belong to the genus *Candida* are these isolates does not produce ascospore, forms pseudohypha with blastospores that appear in pseudohypha branching.Vegetativereproduction with multilateral budding. Colonies are often circular with edges spread or full, forming convex elevations, has no carotenoid pigment,has an ovoid form of cells, are able to ferment some sugars, does not produce urease, forming a pellicle on the surface of the medium and white precipitate

The genus *Candida* is not found in the open sea, but manyof them are found in coastal waters and close to population centers where water is polluted by many domestic waste (Saavedra et al., 1995). Besides that, *Candida* is well known as phosphate solubilizer (Kanti, 2006).

The measurement of soluble phosphate showed that the concentration increased during incubation time in line with the total colony of yeast (Figure 2 and Figure 3). Soluble phosphate concentration can deacreasein a certain incubation time ,because it was consumed by yeast. Soluble phosphate in the medium is used for oxidative respiration activity that plays a role in the transfer or glucose consumption into cells to generate ATP and biomass, that will enhance the growth (Raharjo et al., 2007). In addition, yeast isolates also produce large amounts of phosphate as an excess of nutrient supply that calculated as the concentration of dissolved phosphate. Soluble phosphate concentration of*Candida* W1.1 until 7 days of incubation time is 0.50 ppm and soluble phosphate concentration of *Candida* G3.2 is 0.77 ppm.

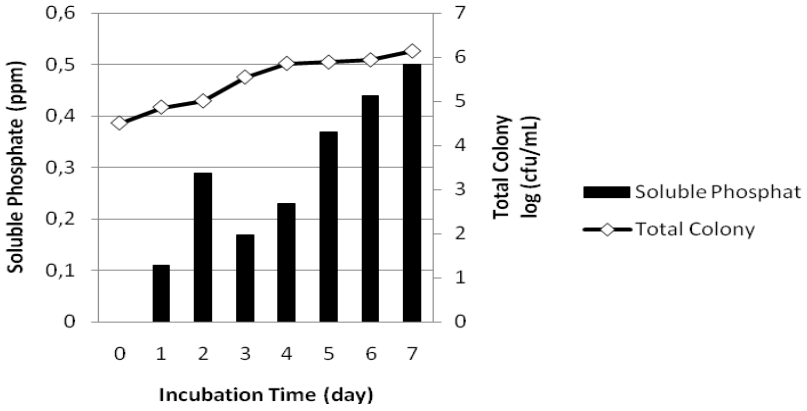

Figure 2. Soluble phosphate concentration and growth curve of *Candida*W1.1

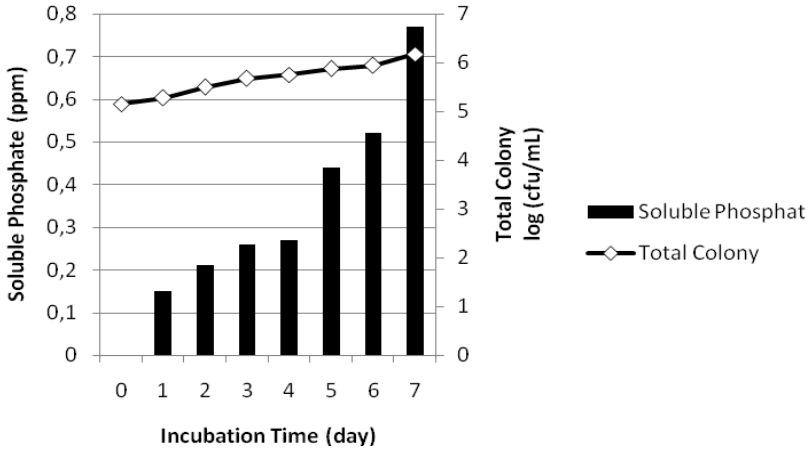

Figure 3. Soluble phosphate concentration and growth curve of *Candida* G3.2

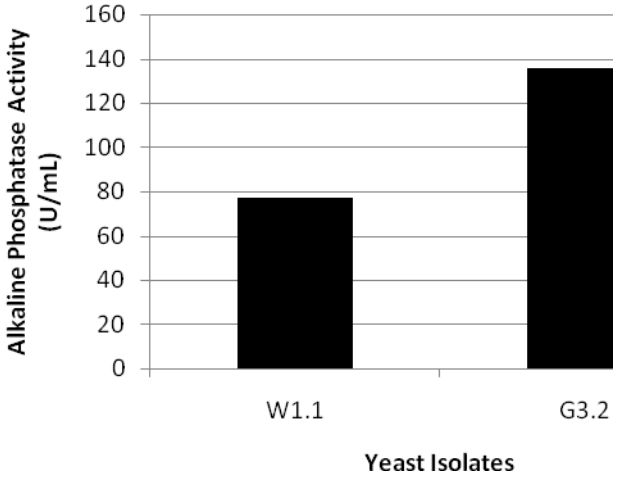

Figure 4. Alkaline Phosphatase Activity of *Candida* W1.1 and *Candida* G3.2

Two of the best yeast isolates fromprevious test (*Candida* W1.1 and *Candida* G3.2) were tested its alkaline phosphatase activity. The test results showed *Candida* G3.2 enzyme activity higher than *Candida* W1.1. *Candida* G3.2 has enzyme activity of 135.8 U / mL while *Candida* W1.1 has enzyme activity of 77 U / mL (Figure 4).

Differences in enzyme activity can occur, even in the same genus. This is due to the synthesis and activity of the enzyme that is influenced by genetic control and distribution of enzymes in the cell (Rogers, 2011). Inorganic phosphates also show the influence of the enzyme synthesisat different strains. One example of the *Candida* genus which is also known to have the potential to produce alkaline phosphatase is *Candidautilis*. *Candidautilis* showed alkaline phosphatase enzyme activity of 0.35 U / mL (Alvarez et al., 1982). Alkaline phosphatase was found in *Candida utilis*, *Candidaalbicans* and *Saccharomyces cerevisiae* derived from intracellular namely the vacuole (Fernandez et al., 1981). Differences in enzyme activity were also influenced by cofactors (activator) in the form of alkaline phosphatase Mg^{2+} or Zn^{2+}. Cofactor that is required depends on the strain of the microorganism used (Dick et al., 2011). The result (Figure 4) get *Candida* G3.2 had higher activity than *Candida* W1.1. *Candida* G3.2 will be optimized to produce the enzyme alkaline phosphatase.

Optimization of incubation time was conducted to determine the best extraction time during the alkaline phosphatase production. The optimum time is determined through the production of enzymes for 7 days with an interval of every 24 hours of extraction to determine the activity of alkaline phosphatase produced. Alkaline phosphatase activity increased with incubation time (Figure 5). The gradual increase in the production of enzymes occur from day-1 and the maximum production obtained on day 3 with the activity of 94.2 U / mL, then seen a decrease in activity on day 4 to day 7. Day 3 can be assumed to be the beginning of the stationary phase. The increased of enzyme activity can be caused by the high metabolic activity of yeast cells in a cell-division and the

synthesis of the enzyme. The secretion of alkaline phosphatase will be increasing along with the number of cells, This is consistent with the statement (Das et al., 2013) that the enzyme secretion depends on the number of cells and the specific microorganism growth phase. The decrease in enzyme activity occurs on day 3 to day 7. This could be caused by the maximum production of the enzyme, there is a production of other products and a decrease in the nutrient medium.

Optimization of the influence of temperature variation and phosphate sources used to obtain optimal temperature and phosphate source that is suitable for the production of alkaline phosphatase. This test was done by incubating the culture at different temperatures (29 °C, 45 °C and 55 °C) using a Pikovskaya medium with phosphate modification. Incubation was performed for 3 days which is obtained from the previous stage. The test results statistically using Two-WayANOVA, the result showed that temperature affects the activity of alkaline phosphatase significantly. While these types of phosphate source did not show a significant difference, but at a temperature of 29 °C and 45 °C the phosphate source of $Ca_3(PO_4)_2$ higher than KH_2PO_4 (Figure 6). The optimal temperature for the production of alkaline phosphatase is at a temperature of 29 °C with the highest activity on both substrates of 97.8 U / mL in $Ca_3(PO_4)_2$ and 93 U / mL in KH_2PO_4. Alkaline phosphatase enzymes can be produced at a temperature range of 29 °C-55 °C. These results similar to some psychrophilic bacteria and*E.coli* that has an optimum temperature at 30 °C-40 °C. Higher temperatures could increase the kinetic energy of the molecules that break down the active bond on the amino group so that the enzyme is denatured (Mahesh, 2010). Temperature plays an important role in producing enzymes of microorganisms because it affects the solubility of oxygen in the nutrient medium, increasing the kinetic energy of the molecules and the speed of enzymatic reactions inside the cell, microorganism growth, and metabolism (Omran and Qaddoori, 2014).Temperature can also affect the sensitivity of yeast to produce

the product concentration, growth rate, fermentation rate, viability, the length of the lag phase and membrane function (Jackson, 2000). Differences in enzyme activity can also be caused by the optimum growth of *Candida* genus at a temperature of 20-38 °C (Komariah and Sjam, 2012).

Among the phosphate sources, $Ca_3(PO_4)_2$ was found suitable for producing alkaline phosphatase (Priya et al., 2014). This is due to inorganic phosphate in alkaline conditions found in the environment in the form of $Ca_3(PO_4)_2$ (Kapri and Tewari, 2010). Besides that, enzymes have the active site. The active site is a specific part of the amino acid side chains that can bind one molecule or several types of molecules when a chemical reaction takes place. Most enzymes show a high degree of specificity for binding specific substrate and catalyze specific reactions to produce specific products (Mahesh et al., 2015). This causes $Ca_3(PO_4)_2$ suitable for the production of alkaline phosphatase compared to other phosphate sources.

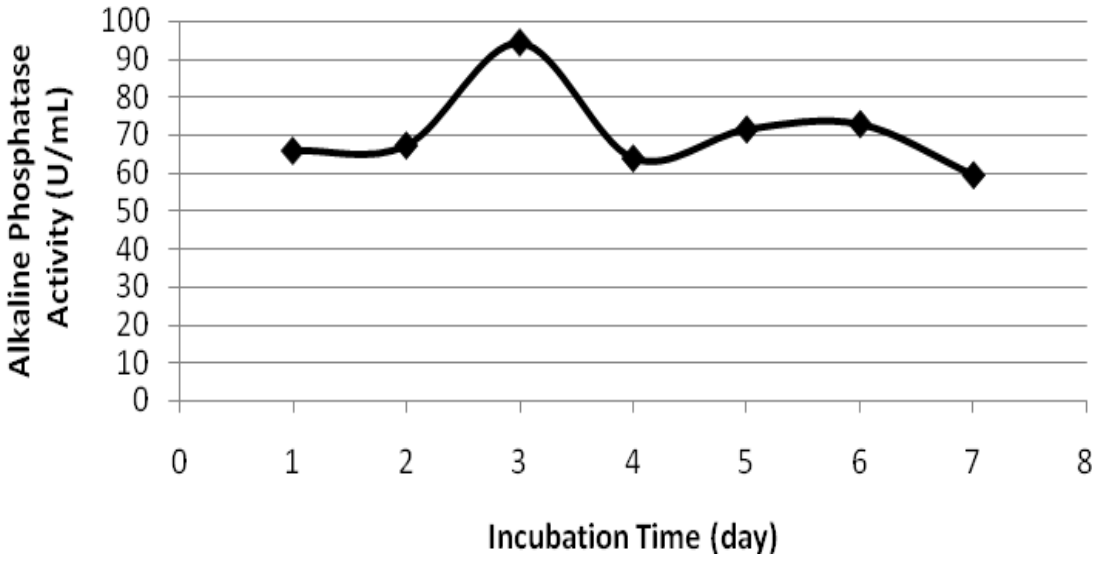

Figure 5. The influence of incubation time on alkaline phosphatase by *Candida* G3.2

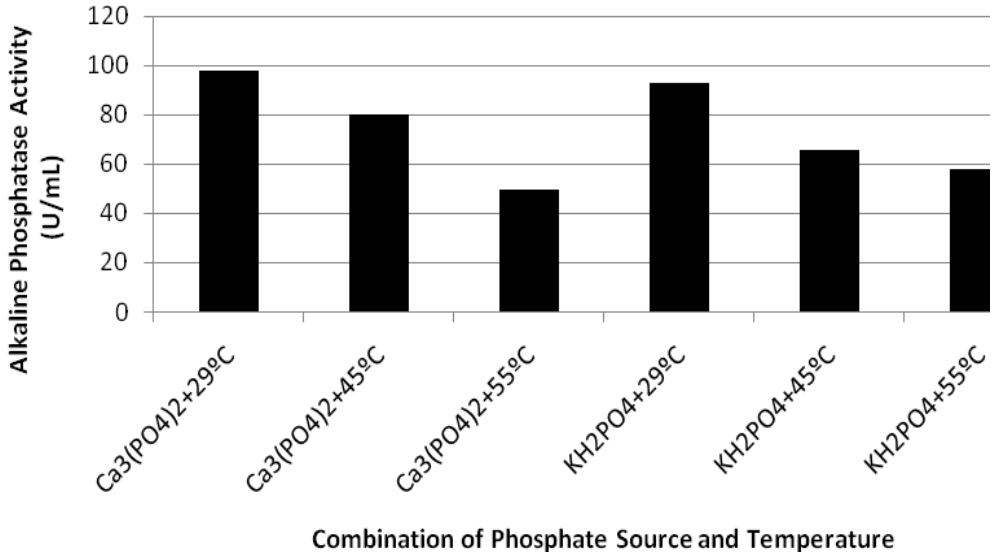

Figure 6. The influence of temperature and phosphate source on alkaline phosphatase activity by *Candida* G3.2

IV. CONCLUSION

The result showed that isolates with the highest phosphate solubilization index were W1.1 and G3.2. The value of W1.1 was 1.18 and the value of G3.2 was 1.15. Yeast with theability to solubilize phosphate was assumed of *Candida*genus.*Candida* W1.1 and *Candida* G3.2 respectively produce soluble phosphate in a concentration of 0.50 ppm at the 7th day and in a concentration of 0.77ppmat the 7th day. *Candida* G3.2 has higher enzyme activity than *Candida* W1.1. *Candida* G3.2 has optimal production at the time of incubation for 3 days, with combination of temperature 29 °C and the source of phosphate used $Ca_3(PO4)_2$ with the value of enzyme activity was 97.8 U/mL

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