Original Research Article

Acid Phosphatases from the Breadfruit *Artocarpus communis* Seeds as Novel Plant Phosphorylating Biocatalysts

ABSTRACT

Aims: Investigation on the phosphotransferase activity of two non-specific acid phosphatases (EC 3.1.3.2) designated as AP1 and AP2, previously isolated from breadfruit (*Artocarpus communis*) seeds for further biotechnological and industrial applications.
Methodology: Transphosphorylation reactions were tested with sodium pyrophosphate as the phosphoryl donor and phenol as its acceptor. Transfer products were quantified by using high performance liquid chromatography.
Results: The two acid phosphatases were able to catalyse phosphoconjugates synthesis using pyrophosphate as the phosphoryl donor and phenol as acceptor. The optimal conditions of transphosphorylation reactions indicated that this synthesis was highly dependent on pH, temperature, time course, donor and acceptor concentrations and enzyme amount. A very short period (1.25 h) was observed for these synthesis reactions catalysed by acid phosphatases isolated from breadfruit (*Artocarpus communis*) seeds. This suggested energy saving during biotransformation processes. The high average yields of 84.20 and 66.78% obtained for AP1 and AP2, respectively, made them useful to phosphorylate a wide range of nucleophile compounds such as nucleotides often used as food additives and pharmaceutical intermediates.
Conclusion: The acid phosphatase AP1 would be the most promising on the basis the better synthesis product yield (84.20%). The two biocatalysts could be considered as new valuable tools for bioprocesses.

Comment [SaA1]: To state the precise applications?

Comment [SaA2]: Include the synthesis method? Concentration of AP1 and AP2? bacteria method? Enzyme?

Keywords: Plant acid phosphatases, transphosphorylation, breadfruit Artocarpus communis seeds, phosphoconjugate synthesis, biocatalysts

1. INTRODUCTION

Transphosphorylation is an efficient process for the large-scale production of new phosphorylated conjugates with improved properties [1, 2, 3]. This reaction is essentially reversible, and the equilibrium position depends on the conditions and the amount of reagents and products present in the reaction mixture [1]. Kinases are well-known phosphorylating enzymes which transfers a phosphate unit from ATP to a variety of acceptors, but the large-scale application is impeded by the need of regenerating ATP and in addition these enzymes are specific for the substrate to be phosphorylated [4]. Nowadays, it has been shown that acid phosphatases (orthophosphoric-monoester phosphohydrolase (EC 3.1.3.2)) catalyzing the hydrolysis of a broad and overlapping range of phosphomonoesters are also able to carry out transphosphorylation reactions in which a phosphate unit is transferred from a donor (phosphomonoesters or pyrophosphate PPi) to an acceptor alcohol [5]. This enzymatic phosphorylation process shows more advantages than

the chemical one that uses phosphoryl chloride ($POCl_3$) [6]. It is well known that enzymes tolerate environmental stress ~~and thanks~~ due to their broad substrate specificity, they are able to accept a wide range of cheap phosphoryl donors and acceptors [6, 7]. In contrast, the chemical methods should be explored with caution due to high number of by-products obtained in typical reactions. Current chemical routes to phosphate esters synthesis often proceed via the corresponding phosphate di or triesters and require harsh reagents and conditions leading to hardly separable product mixtures [8].

Thus, Bacterial phosphatases from the class A nonspecific acid phosphatase family were mostly used, and *Shigella flexneri* [7, 9], *Salmonella enterica* [7], *Morgenella morganii* [5, 1] and *Escherichia blattae* [2] were prominent sources of the enzyme. The phosphoryl transferase activities of nonspecific acid phosphatases from these microorganisms were exploited to produce nucleotides as inosine-5 monophosphate (5-IMP) and guanosine-5 monophosphate (5-GMP) using pyrophosphate (PPi) as a phosphate donor. ~~So, this~~The production of phosphoconjugates is another key area of interest in biotechnology, because they have often used as food additives and as pharmaceutical synthetic intermediates [5].

To ~~our knowledge~~date, there are few reports on the transphosphorylation abilities of plant non-specific acid phosphatases. Nevertheless, due to their wider availability and lesser cost, plant phosphatases could be explored as good substitutes for biotechnological phosphate ester synthesis. ~~Thus,~~ Koffi et al. [3] isolated four non-specific acid phosphatases from the neglected crop *Lagenaria siceraria* seeds exhibiting high phosphoryltransferase activities. In search of new biocatalysts with improved transphosphorylation yield, two acid phosphatases from breadfruit (*Artocarpus communis*) seeds [10] were investigated. In this paper, we report on the phosphoryltransferase activity of these enzymes.

2. MATERIALS AND METHODS

2.1 *Enzymes*

The acid phosphatases AP1 and AP2 (EC 3.1.3.2) used for transphosphorylation reactions originated from the purified enzyme collection of Laboratoire de Biochimie et Technologies des Aliments, UFR Sciences et technologies des Aliments, Université Nangui Abrogoua (Abidjan, Côte d'Ivoire). These phosphatases were purified as described previously [10].

2.2 *Chemicals*

Sodium pyrophosphate (donor), phenol (acceptor) and tyrosine (internal standard) were purchased from Sigma Aldrich. All other reagents used were of analytical grade.

2.3 *Transphosphorylation reactions*

The ability of phosphatases AP1 and AP2 from *A. communis* seeds [10] to ~~catalyse~~ catalyze transphosphorylation reactions was tested with sodium pyrophosphate as the phosphoryl donor and phenol as its acceptor. In typical experiment, transphosphorylation reactions were carried out at 37 °C in a total reaction mixture of 250 µL containing 400 mM of sodium acetate buffer pH 4.0. The reactions were stopped by immersion in boiling water for 3 min, followed by cooling in ice bath. Prior to each HPLC analysis, the reaction mixtures were filtered using Ultrafree-MC filter (0.45 µm) ~~devices~~ (Millipore). Tyrosine was used as the internal standard to correct chromatographic product areas. Twenty microliter (20 µL) aliquots of each reaction mixture always containing the internal standard (2 mM final

Formatted: Subscript

Comment [SaA3]: You manuscript uses US english. Please standardize! Thank you

concentration) were ~~analysed~~ analyzed quantitatively by HPLC. The column used was SPHERECLONE 5 µ??? ODS (2) (250 mm × 4.60 mm; Phenomenex) and phenolic compounds were detected at 254 nm with a SPECTRA SYSTEM UV 1000 detector. The elution was done with a BECKMAN 114 M solvent delivery module pump, at a flow rate of 0.5 mL min^{-1} by using a degassed mixture of acetonitrile/water in the ratio 50:50 (v/v) as mobile phase. The chromatograms were obtained with a SHIMADZU C-R8A CHROMATOPAC V1.04 integrator. The detailed experimental conditions for studying parameters likely to affect the transphosphorylation reactions (pH, time, donor concentration, acceptor concentration and enzyme amount) are given below. For the determination of each parameter, reactions were carried out in triplicate.

Formatted: Superscript

2.4 *Determination of optimum pH*

The pH values were determined at 25°C. For determination of optimum pH, transphosphorylation reactions were performed by incubating at 37°C for 1 h each phosphatase (25 µL) in a pH range of 3.6 to 5.6 (sodium acetate buffer, 400 mM), with 100 mM final concentration of sodium pyrophosphate and 5 mM final concentration of phenol.

2.5 *Determination of optimum time*

To determine the optimum time of transphosphorylation, the optimum pH determined for each enzyme was fixed. The transphosphorylation reactions were performed at different times ranging from 0 to 6 h (at 37°C), in 400 mM sodium acetate buffer at appropriate optimum pH, with 25 µL enzyme, 100 mM final concentration of sodium pyrophosphate and 5 mM final concentration phenol.

2.6 *Determination of phosphoryl donor optimum concentration*

The influence of phosphoryl donor concentrations (0 to 300 mM) on the transphosphorylation reactions was determined under the optimum conditions of pH and time. Phosphatases (25 µL) were separately incubated at 37°C, with different concentrations of sodium pyrophosphate ranging from 0 to 300 mM final concentration and 5 mM phenol.

2.7 *Determination of phosphoryl acceptor optimum concentration*

The influence of phosphoryl acceptor concentrations (0 to 10 mM) on the transphosphorylation reactions was determined under the optimum conditions of pH, time and phosphoryl donor concentration. The phosphatases (25 µL) were separately incubated at 37°C, with different concentrations of sodium pyrophosphate ranging from 0 to 300 mM final concentration and 5 mM phenol.

2.8 *Determination of enzyme amount*

For this study, the optimal condition of pH, time, donor concentration and acceptor concentration determined for each enzyme were fixed. Only the amount of each phosphatase varied from 0 to 10 µg. In the determinations described above, 25 µL of enzyme corresponded to 5 µg, and 2.1 µg of protein, respectively, for AP1 and AP2. All the reactions were stopped by immersion in boiling water for 3 min and the products quantified by HPLC as described in the typical transphosphorylation reaction.

2.9 *Transphosphorylation reactions in optimum conditions*

Ultimately, the optimal conditions of pH, enzyme amount, donor and acceptor concentrations and time were fulfilled to perform a unique transphosphorylation reaction with phosphatases AP1 and AP2 purified from *A. communis* seeds. These reactions were also carried out in triplicate with regard to the typical conditions described in the other experiments, and the synthesized products were quantified by HPLC as described previously.

2.10 *Estimation of the yield of transphosphorylation*

One mol of the synthesized product corresponds to 1 mol of phenol used as acceptor in the reaction mixture. Therefore, the starting phenol concentration deficit at the end of each reaction is typical of the amount of acceptor necessary for the product synthesis. After adjusting areas with the internal standard (tyrosine), transphosphorylation yields were determined as follows: (area of initial phenol – area of remaining phenol)/area of initial phenol × 100.

Comment [SaA4]: Please include the in text citation!

3. RESULTS AND DISCUSSION

The two non-specific acid phosphatases AP1 and AP2 purified from *A.communis* seeds [10] were tested in transphosphorylation reaction of pyrophosphate at pH 4. The retention time of the newly synthesized product was found to be similar to that of phenylphosphate (Data not shown). This shows clearly that the studied enzymes were able to catalyse the phosphorylation of pyrophosphate (PPi) to phenylphosphate. Pyrophosphate was earlier used as donor in many transphosphorylation reactions [3, 11] due to the fact that it is a safe and inexpensive compound which can be used in large excess for various manufacturing processes [12]. Also, this molecule is easily synthesized from phosphate groups [13]. Therefore, an efficient phosphorylation process could be achieved by recycling pyrophosphate to phosphate group by-products for further transfer in transphosphorylation reactions.

Comment [SaA5]: Why? Any reason?

In order to optimize the phenylphosphate formation, five parameters were investigated namely the pH, time, donor concentration, acceptor concentration and enzyme amount dependency. In **Figure 1** the pH dependency of the phosphotransferase reaction is depicted. The highest yield of phenylphosphate (69.25 and 56.75 % respectively for AP1 and AP2) was obtained at pH 3.6. It is noteworthy that there are few reports on phosphotransferase activity of plant acid phosphatases, but it seems that these biocatalysts exhibit better transfer activity at low pHs compared to their hydrolysis activity (optima pHs sited between 5 and 6). A similar behaviour has been observed by KOFFI et al. [3] for four non-specific acid phosphatases isolated from the cucurbit *L.siceraria* seeds. According to Huber et al. [14], the dissimilarity between the optima pHs for phosphorylation and dephosphorylation could be due to ionized groups sited in the active site of the enzymes which would favour synthesis and disadvantage hydrolysis in more acidic conditions.

Formatted: Font: Bold

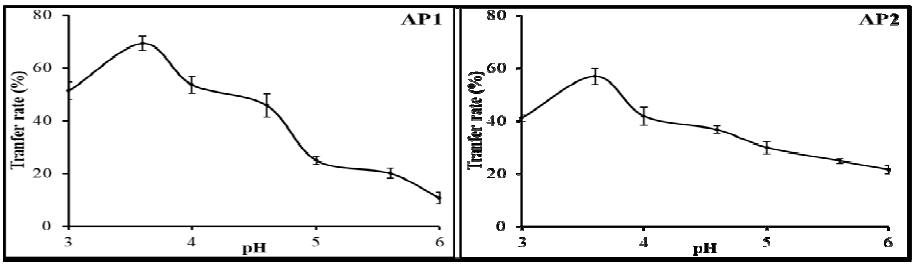

Figure **1.** Effect of pH on transphosphorylation reaction catalyzed by the acid phosphatases (AP1 and AP2) from breadfruit (*A.communis*) seeds.
Bars represent ± SE

The enzyme activities were also measured at various temperatures from 30 to 65°C. AP1 had maximum activity at 40°C whereas AP2 shows maximum activity at 35°C. In these conditions, optima yields of 73.58 and 59.75 % were obtained for phosphatases AP1 and AP2, respectively (**Figure 2**). The optimal temperatures of transphosphorylation were lower than that obtained for dephosphorylation (55°C). So, these temperatures would be advantageous since the studied enzymes have been earlier shown to be stable at temperatures below 55°C [10]. Asano et al. [5]obtained their best transphosphorylation rate at 40°C during the synthesis of Inosine-5'-monophosphate using a phosphorylating enzyme isolated from bacteria *M. morganii*. The general observation would be that both the phosphorylation and dephosphorylation temperatures do not coincide. It appears that the optimum phosphorylation temperature is generally lower (ranging from 30 to 40°C) compared to that of dephosphorylation (above 45°C).

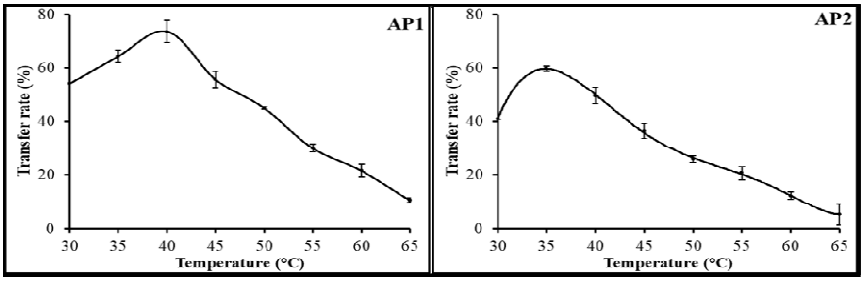

Figure **2.** Effect of temperature on transphosphorylation reaction catalyzed by the acid phosphatases (AP1 and AP2) from breadfruit (*A.*??????????????
Bars represent ± SE

The time course of phenylphosphate synthesis by acid phosphatases AP1 and AP2 isolated from bread fruit *A.communis* seeds is depicted in **Figure 3**. The two enzymes show maximum transphosphorylation yield at 1.25 h. This time course of reaction is very low compared with those obtained for 5'-inosinic acid synthesis as reported by Mihara et al. [2]. These authors have obtained their better phosphotransferase activity at time courses ranging from 18 to 36 h. Ishikawa et al. [12] have reported phosphorylation times sited between 5 and 15 h for inosine-5'-monophosphate (5'-IMP) synthesis. Therefore, the very

Formatted: Font: Not Bold
Formatted: Font: 9 pt
Formatted: Font: Bold
Formatted: Font: Not Bold
Formatted: Font: Bold

short period of synthesis reactions catalysed by the studied acid phosphatases may constitute an interesting feature insofar as this would make it possible to save energy in biotransformation processes using these biocatalysts. Also, it should be remembered that this reaction time is very well included in the stability time (fully active for more than 150 min) of these enzymes as describe by Konan et al. [10].

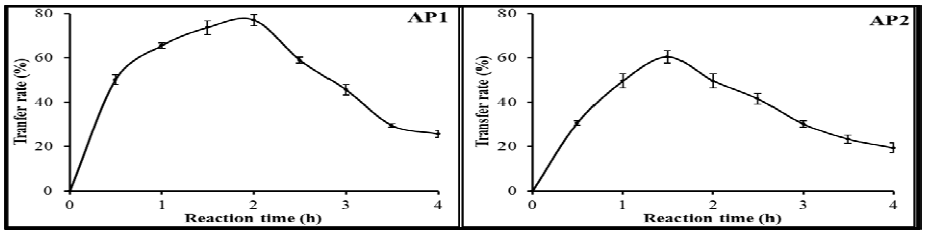

Figure 3. Time course of phenylphosphate synthesis by the acid phosphatases (AP1 and AP2) from breadfruit (*A.communis*) seeds. Bars represent ± SE

The yields of phenylphosphate produced by nonspecific acid phosphatases AP1 and AP2 were highly dependent on donor and acceptor concentration (**Figures 4 and 5**). Similar behaviours were observed by Van Herk et al. [9]. In our study, the maximum concentrations of phosphorylated product were obtained from 200 mM pyrophosphate and 12.5 mM phenol for both AP1 and AP2. In these conditions, optimum yields of 82.39 and 65.71% were obtained for AP1 and AP2, respectively. As presented in figures 4 and 5, it seems that higher concentrations of donor and acceptor than those obtained in this study would inhibit the phosphoryltransferase activities. This would explain the decrease in the transfer rate since it is well known that high substrate loads are required to achieve high product titers, while kinetic control is crucial to keep the phosphorylation/hydrolysis ratio as high as possible due to the competition between hydrolysis and transphosphorylation in aqueous medium [15]. A common mechanistic feature of phosphate-transferring phosphatases is the formation of a covalent phospho-enzyme intermediate, which undergoes either nucleophilic attack by water (hydrolysis) or accepts a suitable nucleophile (transphosphorylation).

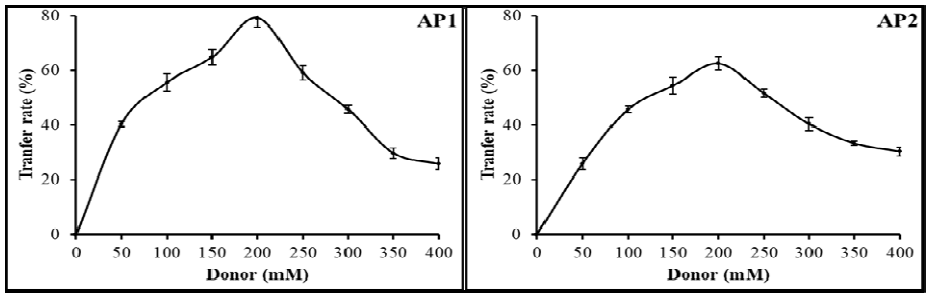

Figure 4. Effect of phosphoryl donor (sodium pyrophosphate) on transphosphorylation reaction catalyzed by the acid phosphatases (AP1 and AP2) from breadfruit (*A.communis*) seeds. Bars represent ± SE

Formatted: Font: Not Bold

Formatted: Font: Bold

Formatted: Font: Not Bold

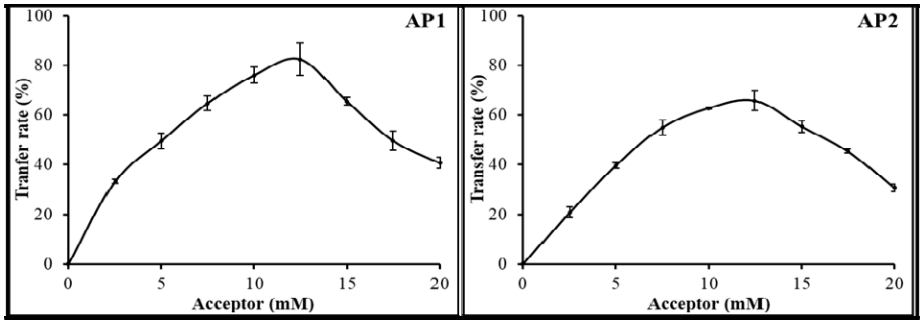

Figure 5. Effect of phosphoryl acceptor (phenol) on transphosphorylation reaction catalyzed by the acid phosphatases (AP1 and AP2) from breadfruit (*A.communis*) seeds. Bars represent ± SE.

Figure 6 shows effect of enzyme amounts on transphosphorylation reactions. This result indicates that high amounts of enzymes would be unfavourable to the phosphoryl transfer reaction. It appears that these high enzyme amounts result in the release of high amounts of inorganic phosphate, which could inhibit the phosphatase [16]. The phosphotransferase activities were optimal with enzyme amounts of 6 µg for both AP1 and AP2, and average yields of 83.60 and 65.10 % were respectively obtained.

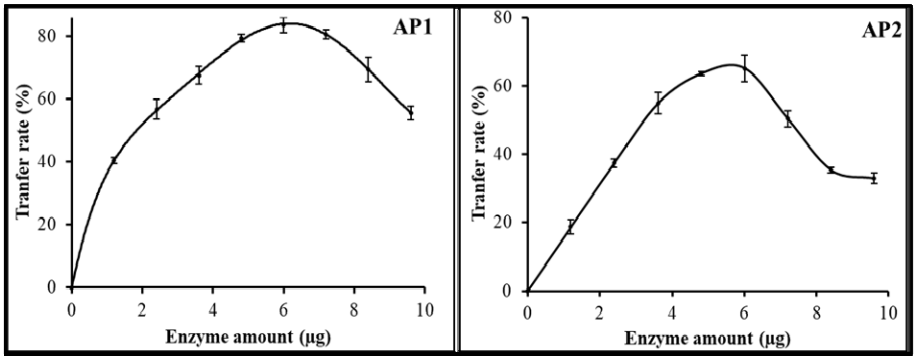

Figure 6. Effect of enzymatic unit on transphosphorylation reaction catalyzed by the acid phosphatases (AP1 and AP2) from breadfruit (*A.communis*) seeds. Bars represent ± SE.

Table 1 summarized the average yields of transfer at the different stages as well as those obtained under the optimal conditions. During this study, yields significantly improved (about 10 to 15 %). In optimal conditions yields of 84.20 and 66.78 % were obtained for AP1 and AP2, respectively. These values are higher than those reported for nonspecific acid phosphatases from seeds of the orphan crop *L. siceraria* [3] and for the nonspecific acid phosphatase from *Salmonella typhimurium* LT2 [17]. Although they are plant acid phosphatases, the studied enzymes exhibit good transphosphorylation potentials compared to bacterial phosphoryl transfer enzymes which were often modified to improve their transferase properties. Regarding these interesting phosphoryltransferase activities, it is likely that nonspecific acid phosphatases from the breadfruit *A. communis* seeds would be

Formatted: Font: Not Bold

Formatted: Font: Bold

Formatted: Indent: First line: 0"

Formatted: Font: Not Bold

Formatted: Font: Bold

useful to phosphorylate a wide range of nucleophile compounds such as nucleotides often used as food additives and pharmaceutical intermediates.

Table 1. Transfer product yields in optimal conditions of the transphosphorylation assay catalyzed by acid phosphatases AP1 and AP2 from breadfruit (*A.communis*) seeds.

Parameters	Transphosphorylation yield	
	AP1	AP2
pH	69.25 ± 0.8	56.75 ± 1.1
Temperature	73.58 ± 0.7	59.75 ± 2.2
Reaction time	76.98 ± 1.2	60.50 ± 0.9
Donor	79.08 ± 1.5	62.47 ± 0.9
Acceptor	82.39 ± 0.5	65.71 ± 0.7
Enzyme unit	83.60 ± 0.9	65.10 ± 1.0
Optimum conditions	84.20 ± 0.6	66.78 ± 0.5

Values given are the average from at least three experiments.

4. CONCLUSION

The data obtained in this study showed that acid phosphatases from seeds of the breadfruit *A.communis* may be good alternative biocatalysts to form phosphoconjugates by applying them in synthesis of various phosphorylated compounds such as nucleotides often used as food additives and pharmaceutical intermediates. Shorter time course reactions (1.25 h at the most) were observed for optimum synthesis, suggesting energy saving during biotransformation processes. The acid phosphatase AP1 would be the most promising on the basis the better synthesis product yield (84.20 %). The two biocatalysts could be considered as new valuable tools for bioprocesses.

REFERENCES

[1] Mihara Y, Utagawa T, Yamada H, Asano Y. Acid phosphatase/ phosphotransferases from enteric bacteria. J BiosciBioeng2001; 2:50-54.

[2] Mihara Y, Ishikawa K, Suzuki E, Asano Y. Improving the Pyrophosphate-inosine Phosphotransferase Activity of *Escherichia blattae* Acid Phosphatase by Sequential Site-directed Mutagenesis. BiosciBiotechnolBiochem 2004; 68(5): 1046-1050.

[3] Koffi DM, Faulet BM, Gonnety JT, Bédikou ME, Kouamé LP, Zoro Bi IA,Niamké SL. Novel Plant Acid Phosphatases from the Orphan Crop *Lagenariasiceraria*(Molina) Standl. for Phosphate Ester Synthesis. Philip Agric Scientist 2012; 95(1): 14-21.

[4] Faber K. *Biotransformation in organic chemistry* 2000; pp 123–134, 4th edition, Springer, Berlin.

[5] Asano Y, Mihara Y, Yamada H. A novel selective nucleoside phosphorylation enzyme from *Morganella morganii*. J BiosciBioeng1999; 87: 732-738.

[6] Yoshikawa M, Kato T, Takenishi T. Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. Bull Chem Soc Jpn 1969; 42: 3505-3508.

[7] Tanaka N, Hasan Z, Hartog AF, Van Herk T, Wever R, Sanders RJ, Phosphorylation and dephosphorylation of polyhydroxy compounds by class A bacterial acid phosphatases. Org Biomol Chem 2003;1: 2833-2839.

[8] Tasnádi G, Lukesch M, Zechner M, Jud W, Hall M, Ditrich K, Baldenius K, Hartog AF, Wever R, Faber K. Exploiting Acid Phosphatases in the Synthesis of Phosphorylated Monoalcohols and Diols. Eur J Org Chem 2016; 2016(1): 45-50.

Formatted: Font: Not Bold

Comment [SaA6]: Kindly check again and follow the Journal's format!

[9] Van Herk T, Hartog AF, Van Der Burg AM, Wever R. Regioselective phosphorylation of carbohydrates and various alcohols by bacterial acid phosphatases; probing the substrate specificity of the enzyme from *Shigella flexneri*. Adv Synth Catal 2005; 347: 1155-1162.

[10] Konan HK, Yapi DYA, Yué Bi CY, Koné TFM, Kouadio PEJN, Kouamé LP. Biochemical ~~Characterization~~ characterization of ~~Two~~ two ~~Acid~~ acid ~~Phosphatases~~ phosphatases Purified from Breadfruit (*Artocarpuscommunis*) Seeds. Chiang Mai J Sci 2016; 43(5): 1102-1113.

[11] Babich L, Peralta JLVM, Hartog AF, Wever R. Phosphorylation by Alkaline Phosphatase: Immobilization and Synthetic Potential. Int J Chem 2013; 5(3): 87-98.

[12] Ishikawa K, Mihara Y, Shimba N, Ohtsu N, Kwasaki H, Suzuki EI, Asano Y. Enhancement of nucleoside phosphorylation activity in an acid phosphatase. Protein Eng 2002; 15(7): 539-543.

[13] Staffel T. Condensed sodium phosphates 1991. Weinheim: VCH Verlag.

[14] Huber RE, Gaunt MT, Sept RL, Babiak MJ. Differences in the effects of pH on the hydrolytic and transgalactosylic reactions of beta-galactosidases (*Escherichia coli*). Can J Biochem Cell Biol 1983; 61: 198-206.

[15] Mihara Y, Utagawa T, Yamada H, Asano Y.Phosphorylation of nucleosides by the mutated acid phosphatase from *Morganella morganii.* Appl Environ Microbiol2000; 66: 2811-2816.

[16] Fernley HN, Walker PG. Studies on alkaline phosphatase. Inhibition by phosphate derivatives and substrate specificity. Biochem J 1967; 104: 1011-1018.

[17] Dissing K, Uerkvitz W. Class B nonspecific acid phosphatase from *Salmonella typhimurium* LT2 phosphotransferase activity, stability and thiol group reactivity. Enzyme Microb Technol 2006; 38: 683-688.

Comment [SaA7]: Check format

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Comment [SaA8]: What is this source?????