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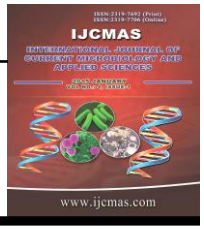


**Review Article**

# Microbial alkaline phosphatases in bioprocessing

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**A B S T R A C T**

Microbial alkaline phosphatases are produced on large scale in an economical way within limited space and in short time. Alkaline phosphatases are crucial in phosphate metabolism. The enzyme with its wide specificity and activity is potential in bioprocessing. An emphasis is placed on the diverse applications of microbial alkaline phosphatases. Alkaline phosphatase is the most commonly used enzyme in immunoassays. Most of the microbial alkaline phosphatases of significant application in diagnostic studies are obtained from bacteria. Alkaline phosphatase based biosensors play an essential role in environmental monitoring. Microbial alkaline phosphatases have a major application as biofertilizer. They are also useful for the evaluation of the soil quality and the perturbation occurring in agricultural fields. The assessment of alkaline phosphatase activity is used as a marker for milk pasteurization in dairy industries. Alkaline phosphatase is an important biochemical tool in limnological studies.

## Introduction

Enzymes are vital to life where life cannot exist without them. Enzymes produced by living cells are giving newer life to them. They have been used directly and indirectly by mankind since the dawn of history. Unlimited varieties of enzymes are available from microorganisms found in diverse and extreme conditions.

Microorganisms producing alkaline phosphatases are wide spread in nature. Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) is a hydrolase enzyme functioning at alkaline pH. The metalloenzyme containing the two zinc ions are involved in catalysis whereas the magnesium ion is important in structural stabilization (Simpson *et al*., 1968; Anderson *et al*., 1975). The enzymatically active alkaline phosphatase hydrolyzes phosphates from many types of molecules like nucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. Alkaline phosphatases play an indispensable role in phosphate metabolism and the production of alkaline phosphatase is regulated by the phosphoester compounds available in the

environment (Nalini *et al*., 2014). Divalent metal ions are required for the activity of alkaline phosphatase while chelators such as EDTA inhibit the alkaline phosphatase activity (Cembella *et al*., 1982).

Alkaline phosphatase of *Escherichia coli* is the most studied prokaryotic alkaline phosphatase (Wanner, 1987). Bradshaw *et al*. (1981) determined the complete aminoacid sequence of alkaline phosphatase monomer. Biosynthesis (Derman and Beckwith, 1991; Karamyshev *et al*., 1998), structure and catalytic properties (Coleman, 1992) of *Escherichia coli* alkaline phosphatase has been extensively studied. Studies on bacterial alkaline phosphatases have been less focused even though bacteria are known to have significant alkaline phosphatase activities (Martinez and Azam, 1993). Several alkaline phosphatases have been investigated from microorganisms. Alkaline phosphatases having high thermostability were described from different strains of bacteria and filamentous fungi such as *Thermotoga neapolitana* (Dong and Zeikus, 1997), *Thermus thermophilus* (Pantazaki *et al*., 1998), *Thermus caldophilus* (Park *et al*., 1999), *Bacillus stearothermophilus* (Mori *et al*., 1999), *Bacillus licheniformis* (Pandey and Banik, 2011), *Humicola grisea* var. *thermoidea* (Buainain *et al*., 1998) and *Scytalidium thermophilum* (Guimarães *et al*., 2001).

## Alkaline phosphatases in bioprocessing

Alkaline phosphatases are of significant headway in scientific and bioindustries. The world enzymes demand is increasing at an Average Annual Growth Rate (AAGR) of 6.3 percent, reaching a value of nearly $7 billion by 2017 (World Enzymes, 2014). In the world market of $100 million, alkaline phosphatase is having biggest share of $20 million. Use of microbial alkaline phosphatases in different fields is constantly increasing. Some microbial alkaline phosphatases have thermostability and high catalytic properties, suitable for various biotechnological applications (Guimarães *et al*., 2003). Bacterial alkaline phosphatase is usually used in biomedical research and industry as it is relatively resistant to denaturation, degradation and inactivation (Reid and Wilson, 1971). Commercial preparations of alkaline phosphatase are more commonly obtained from *Escherichia coli* (Seeburg *et al*., 1977). Alkaline phosphatases of filamentous fungi with other advantageous properties may be of interest in bioprocessing (Pereira *et al*., 1995). The applications of alkaline phosphatases in diverse areas such as molecular biology, immunology, diagnostics and dairy technology have been well documented (Wels *et al*., 1992; Zueva *et al*., 1993**;** Suzuki *et al*., 1999; Rankin *et al*., 2010).

**Genetic engineering and molecular biology:** Alkaline phosphatases play a vital role in DNA sequencing analysis and molecular cloning (Suresh and Das, 2014). It hydrolyzes phosphate groups from monophosphate esters and oligonucleotides. The enzyme is specific to 5'-nucleotidases (Ammerman and Azam, 1985), hence used in dephosphorylation of 5'-phosphorylated nucleic acids. Alkaline phosphatase is a valuable enzyme in expression and fingerprinting studies for labeling of DNA and RNA fragments (Githui *et al*., 1999; Boulain and Ducancel, 2004). The enzyme is also involved in removing phosphate groups from proteins (Sitdhipol *et al*., 2012). The biotechnological potential of these enzymes has received considerable attention in recombinant DNA technology (Pereira *et al*., 1995). *Escherichia coli* alkaline phosphatase is classically used in the field of

molecular biology (Zueva *et al*., 1993) to remove the terminal monoesterified phosphate from ribo-oligonucleotides and deoxyribo-oligonucleotides (Wilson *et al*., 1964; Reid and Wilson, 1971). Heat labile alkaline phosphatases are important enzymes in biomedical research as they can be easily inactivated after its specified application (Lu *et al*., 2010). Antarctic bacteria produced heat labile alkaline phosphatases and were effective in rapid 5'-end labeling of nucleic acids in comparison with commercial alkaline phosphatases (Kobori *et al*., 1984; Rina *et al*., 2000). Alkaline phosphatase of *Cobetia marina* is potential in the introduction of labeled $^{32}P$ into 5' ends of DNA fragments (Yu Plisova *et al*., 2005).

Alkaline phosphatases are widely used in the construction of recombinant plasmids (Zappa *et al*., 2001). Alkaline phosphatase treated plasmid vectors in cloning experiments prevents the recircularization and dimerization of the plasmid (Pereira *et al*., 1995). The enzyme removes the 5'-phosphate group of linearized plasmid DNA without hydrolyzing the DNA molecule (Guimarães *et al*., 2007) indicating its potential use in recombinant DNA protocols as suggested by Pereira *et al*. (1995) for *Neurospora crassa* alkaline phosphatase activity. Properties of thermostable alkaline phosphatase obtained from *Geobacillus thermodenitrificans* T2 is suitable for molecular cloning applications (Zhang *et al*., 2008). Highly active bacterial alkaline phosphatase variants are used as reporter enzymes (Sadeghi *et al*., 2005). In gram positive microorganisms the alkaline phosphatase reporter transposon is used for the identification of genes encoding secreted proteins (Gibson and Caparon, 2002). The enzyme labeled oligonucleotide probes are used as culture confirmation reagents for the rapid identification of microorganisms (Glover and Harris, 1998).

**Clinical diagnosis:** Alkaline phosphatase is the most commonly used enzyme in enzyme-labeled antigens and antibodies of enzyme immunoassay and enzyme-linked immunosorbent assay to detect the biological molecules (Gan and Patel, 2013). Antibodies directed against a specific bacterial alkaline phosphatase antigen were detected in acute bacterial infections (Ritter *et al*., 1997). *Escherichia coli* alkaline phosphatase which exhibits substrate specificity without loss of activity was efficiently expressed in *Escherichia coli* when coupled to different antigens (Gillet *et al*., 1993; Chanussot *et al*., 1996) or antibody fragments (Muller *et al*., 1999; Mousli *et al*., 2007). Alkaline phosphatase has a major application in immunodetection (Suzuki *et al*., 1999). Bacteria were identified based on the quantification of bound alkaline phosphatase-labeled anti-DNA-RNA to the DNA-RNA hybrids (Miller *et al*., 1988). Bel-Ochi *et al*. (2013) designed a recombinant SAG1 gene genetically fused to *Escherichia coli* alkaline phosphatase for use in *Toxoplasma* serodiagnosis tests to detect antibody responses against *Toxoplasma gondii*. Alkaline phosphatases are used in the preparation of Ig-enzyme conjugates for immunologic assays (Yu Plisova *et al*., 2005). Recombinant antibody-alkaline phosphatase conjugates can be used in diagnosis of human immunoglobulins, as exemplified in the case of anti-hepatitis B immunoglobulin (Carrier *et al*., 1995). Hoffman and Wright (1985) reported the identification of exported proteins in various bacterial systems from the fusion of secreted proteins to alkaline phosphatase. Alkaline phosphatase fusion protein which binds to erbB-2 protein can be detected directly on tumor cells using a substrate for alkaline phosphatase (Wels *et al*., 1992). Oliaro *et al*. (2000) detected *Helicobacter pylori*

exported proteins with the application of alkaline phosphatase fusion methodology.

**Biosensors:** The use of alkaline phosphatase biosensors in the detection of pesticides or heavy metals has been studied. Tekaya *et al*. (2013) reported that cyanobacterium, *Arthrospira platensis* can be able to produce reporter alkaline phosphatase. Cyanobacteria cell containing alkaline phosphatase was used as reporting enzyme in *Anacystis nidulans* for monitoring the heavy metal toxicity (Awasthi, 2012). Alkaline phosphatase of *Chlorella vulgaris* extracellular membrane acts as reporter element in the presence of heavy metals (Chouteau *et al*., 2005).

Heavy metals were detected from inhibition of alkaline phosphatase present on *Chlorella vulgaris* microalgae (Durrieu and Tran-Minh, 2002). Ionescu *et al*. (2006) developed an electrochemical biosensor on a platinum electrode and the alkaline phosphatase activity was monitored by the oxidation current of *p*-nitrophenol. The oxidation current response decreased linearly with increasing concentration of heavy metals in chronoamperometry experiments (Chong *et al*., 2008). Singh and Mittal (2012) constructed an amperometric biosensor to determine mercury with glassy carbon electrode and the electrode showed its selectivity for silver, alkali metals, alkaline earth metals and transition metals. Guedri and Durrieu (2008) designed a conductometric biosensor based on the measurement of alkaline phosphatase activity to monitor aquatic environments. The changes in conductivity induced by the catalytic reaction of the enzyme exposed to Cd were detected with conductometric electrodes (Chouteau *et al*., 2004; Chouteau *et al*., 2005). Conductometric micro transducer was used to detect Cd, Cu, Ni, Pd and Zn ions (Berezhetskyy *et al*., 2007).

**Dairy industries:** Most of the enzymes activity is diminished or inactivated in milk pasteurization. The assessment of alkaline phosphatase activity is used as a marker for adequate pasteurization of milk (Harding, 1991; Fenoll *et al*., 2002; Rankin *et al*., 2010). Kay and Graham (1935) developed the first enzymatic test for detecting the efficiency of pasteurization based on the inactivation of alkaline phosphatase. Microbial alkaline phosphatases are considerably thermal resistant than milk alkaline phosphatases. Alkaline phosphatase activity has been identified in pasteurized milk as a result of contamination with bacterial alkaline phosphatases (Hammer and Olson, 1941). Microorganisms such as *Bacillus anthracis, Bacillus cereus, Bacillus megaterium* (Dobozy and Hammer, 1969), *Saccharomyces cerevisiae* (Gorman and Hu, 1969) and *Micrococcus sodonensis* (Glew and Heath, 1971) produce both heat labile and heat stable alkaline phosphatases. Pasteurization destroys the most heat resistant pathogens *Coxiella burnetii* and *Mycobacterium tuberculosis* found in raw milk (Cerf and Condron, 2006). Pasteurization process incorporating homogenization resulted in greater inactivation of the bacteria, *Mycobacterium avium paratuberculosis* (Grant *et al*., 2005).

The detection of elevated alkaline phosphatase levels is a common procedure for milk quality control to determine whether milk has been sufficiently pasteurized or there is a post pasteurization contamination with raw milk (Aschaffenburg and Mullen, 1949; Murthy and Cox, 1988; Stabel, 2003; Payne and Wilbey, 2009). Alkaline phosphatase tests are considered to be important to prevent the tracing of pathogenic bacteria like *Campylobacter jejuni, Listeria monocytogenes* and *Salmonella dublin* (C.D.C., 1984; C.D.C., 1986; Hayes *et al*.,

1986). Alkaline phosphatase activity is high in milk of subclinical mastitis infected cows than the alkaline phosphatase activity observed in non-infected milk (Babaei *et al*., 2007; Matei *et al*., 2010). Heat resistant microbial alkaline phosphatases yielding negative results to alkaline phosphatase activity test is suggested to test again after laboratory repasteurization (Knight and Fryer, 1989). Murthy and Kaylor (1990) recommended the differentiation of microbial alkaline phosphatases from residual alkaline phosphatase by agarose-gel electrophoretic technique. Later the immunological development of polyclonal antibodies produced against raw milk alkaline phosphatase is used to detect milk alkaline phosphatase from interference with microbial alkaline phosphatases, antibiotics or pesticides (Vega-Warner *et al*., 1999).

Alkaline phosphatase methodology is also important in sampling of cheese in dairy industries. Bacteria are used as a part of manufacturing process in milk products like cheese and butter (Walstra *et al*., 2006). Initial enzyme levels and the involved thermal procedure in processing are related to the residual levels of the enzyme (Soares *et al*., 2013).

Facultative heterofermentative bacteria such as enterococci, lactobacilli, micrococci and propionibacteria were found in higher levels in cheese prepared from raw milk (Grappin and Beuvier, 1997), responsible for the production of alkaline phosphatases. Different technological steps and composition of cheese affects the detection limits of alkaline phosphatase (Harding, 1991; Yoshitomi, 2004; Harding and Garry, 2005). Rosenthal *et al*. (1996) demonstrated that alkaline phosphatase levels increased with storage time in a variety of cheese samples due to the growth of *Penicillium roqueforti*.

**Agroecosystem:** The majority of agricultural soils are large reserves of phosphorus of which a considerable part has accumulated as a result of regular applications of phosphate fertilizers (Richardson, 1994). Alkaline phosphatase activity by soil amendments increases the availability of phosphorus to plants (Nalini *et al*., 2014). Alkaline phosphatase activity is affected by agricultural practices such as tillage and residue management (Deng and Tabatabai, 1997). Wang *et al*. (2011) reported the increase of alkaline phosphatase activity in non-till treatments with an increase of residue input amounts. Alkaline phosphatase activities were studied in acid farmfields and alkaline farmfield soils (Eivazi and Tabatabai, 1977). Evaluation of microbial alkaline phosphatase is used as an indicator to determine the soil quality in agricultural fields (Jordan *et al*., 1995). Cyanobacterium, *Anabaena oryzae* alkaline phosphatase has a potential application as biofertilizer in high salinity and alkaline soils (Singh *et al*., 2006). Dick *et al*. (2000) investigated the use of alkaline phosphatase activity as pH adjustment indicator in determining the optimum soil pH required for crop production. Measurement of alkaline phosphatase activity has been considered to assess the perturbation occurred due to the introduction of genetically modified microorganisms in the ecosystem (Naseby and Lynch, 1998). Effect of transgenic plants on soil metabolism has been studied by the determination of alkaline phosphatase activity (Donegan *et al*., 1999). Alkaline phosphatase of mycorrhizal colonization has been suggested as a marker for analyzing the symbiotic efficiency of root colonization (Tisserant *et al*., 1993).

**Aquatic ecosystem:** Alkaline phosphatases are prominent enzymes in aquatic environment. The occurrence of alkaline

phosphatases in aquatic and sewage systems has been studied (Reichardt *et al*., 1967; Berman, 1970; Jones, 1972; Flint and Hopton, 1977). Assessment of alkaline phosphatase activity of microorganisms is considered as an important biochemical tool in limnological studies (Pandey and Parveen, 2011). The extracellular alkaline phosphatase dissolved in natural waters is believed to play a role in nutrient dynamics (Berman, 1969; Kobayashi *et al*., 1984). López *et al*. (2006) determined that alkaline phosphatases of lake water samples are contributing significantly to the phosphate pool. Alkaline phosphatase activity is one of the most survival strategies for growth and survival of cyanobacteria under phosphate deficiency conditions (Bhaya *et al*., 2000). Sebastian and Ammerman (2009) investigated the potential role of marine bacterial alkaline phosphatases in the environment. Alkaline phosphatases have significant ecological implications in the transport and intracellular hydrolysis of organophosphate molecules in marine biota (Luo *et al*., 2009). Marine bacteria induce alkaline phosphatase in insufficient inorganic phosphate and therefore bulk alkaline phosphatase activity measurements have been used to estimate the phosphorus status of microbial communities (Cotner *et al*., 1997; Van Wambeke *et al*., 2002). Cotner and Wetzel (1991) analyzed alkaline phosphatase in the eutrophic and oligotrophic zones and suggested the functions of alkaline phosphatases in the processes of phosphorus regeneration in the eutrophic zone. The alkaline phosphatase activity can be used as determinant of phosphorus deficiency status in phytoplankton communities (Labry *et al*., 2005). Measurement of alkaline phosphatase activity has been used as biomarker in determining chemical pollution in freshwater ecosystems (Durrieu *et al*., 2003).

**Other uses:** Some other advantageous properties of alkaline phosphatases for practical applications have been revealed. Alkaline phosphatase serves as a prototype for a wide variety of enzymes utilizing two metal ions for phosphoryl transfer reactions (Beese and Steitz, 1991; Steitz, 1999). The activity of alkaline phosphatase is important in the pharmaceutical and food industries (Niehaus *et al*., 1999; Vieille and Zeikus, 2001). Alkaline phosphatase is used in the manufacture of antitumor compound to convert etoposide phosphate to etoposide (Politino *et al*., 1996). Bacterial alkaline phosphatase fusion system can be used to screen for inhibitory compounds from various plant extracts (Yamabhai, 2005). The activity of alkaline phosphatase acts as more appropriate indicator for assessment of quantity of crude oil pollution in crude oil polluted agricultural soil (Ohiri *et al*., 2013). *Bacillus flexus* alkaline phosphatase can be used as bioremediation agent to detoxify and mineralize environmental contaminants like xenobiotic organophosphates (Falguni and Sharma, 2014). Alkaline phosphatase has an efficient application in biological wastewater treatment processes (Xie *et al*., 2010). The utility of alkaline phosphatase was extended to metal phosphate precipitation. Chaudhuri *et al*. (2013) evaluated the precipitation of heavy metals from single-ion solutions as well as industrial effluents using bacterial alkaline phosphatase.

Many scientific and industry entities recognized the utility of microbially produced alkaline phosphatases. Microbial alkaline phosphatases of their widely existence, activity and stability are potential in biomethodologies. The rapid exploitation of novel developments of microbial alkaline phosphatases in bioprocessing is substantial.

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