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Optimization of production of alkaline phosphatase by a facultative alkaliphile *Bacillus flexus* FPB17 isolated from alkaline lake soils

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A facultative alkaliphilic bacterium, *Bacillus flexus* FPB17 (GenBank accession no. JN415115), isolated from alkaline lake soils from North Gujarat, India, was studied for the production of alkaline phosphatase enzyme. The optimum conditions for the enzyme production were pH 9, temperature 35 ºC, fermentation period 48 h, inoculum volume 2% v/v, shaker speed 140 rpm. Non aqueous system was observed to repress the enzyme production. Deviations from optimum fermentation parameters drastically disturbed the enzyme production. The alkaline phosphatase productivity was not growth associated and was found to be optimum under aerobic, mesophilic, alkaliphilic conditions.

Keywords: Alkaliphilic, *Bacillus flexus* FPB17, Alkaline phosphatase, growth associated productivity

Introduction

Alkaline phosphatases (ALPs; E.C.3.1.3.1) are nonspecific, phosphomonoesterases, metalloenzymes existing in various organisms (Posen, 1967; Simao *et al*., 2007; Junior *et al.,* 2008) that hydrolyze a wide variety of phosphate esters and are classified according to optimum pH ranging from 7.5 to 11.0 (Dhaked*et al.,* 2005; Gong *et al.,* 2005). Phosphatases are crucial for survival of organisms to provide inorganic phosphate (Pi) nutrition for synthesis of nucleic acids, phosphorylated sugars and proteins, etc (Prada *et al*., 1996; Zappa *et al.,* 2001). Phosphatases show great structural and functional diversity with respect to subunit size, metal ion requirements and substrate specificities etc. (Prada *et al*., 1996). ALP is mostly extracellular as in *Bacillus* sp. RK11 worked out by Kelly *et al.,* 1984. The importance of ALPs in

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diagnostics, immunology, clinical medicine and molecular biology has made them popular in scientific studies and commercial utility (Jablonski *et al*., 1986; Ishii and Ghosh, 1993; Plebani *et al.,* 1996; Engvall and Perlman, 1997; Suzuki *et al*., 1999; Chen *et al*., 2006; Muginova *et al.,* 2007; Sun *et al.,* 2007; Nilgiriwala *et al*., 2008). It has been conventionally used as an index of adequate pasteurization, and the detection of ALP activity of thermally treated liquid milk products has become a common procedure for milk quality control (International Dairy Federation, 1991). The present work deals with the optimization of cultural parameters for production of extracellular alkaline phosphatase by facultative alkaliphile *Bacillus flexus* FPB17 isolated from alkaline lake soils from North Gujarat.

Materials and methods

Culture and production medium conditions

Bacillus flexus FPB17, used in the study, was an isolate from the sediment sample of an alkaline lake located in Bhilot village, Radhanpur town, District Patan. The strain was maintained on nutrient agar slants containing 1% peptone, 1% meat extract, 0.5% NaCl and 2% agar. ALP production and inoculum preparation was carried out in nutrient broth (N. broth) containing 1% peptone, 1% meat extract, 0.5% NaCl with initial pH 9.0. Inoculum was developed by transferring single colony from the grown culture in 25 ml N. broth in 100 ml Erlenmeyer flask, incubated on an orbital shaker at 35 °C and 120 rpm for 6 h was used to achieve optical density in the range of 0.8-1.2 at 600 nm. 2% v/v inoculum was transferred to 50 ml of N. broth in 250 ml Erlenmeyer flask and incubated at 35 °C, 120 rpm for 48 h unless specified otherwise.

Optimization of parameters for production of ALP

The effect of parameters *viz.* pH (5–13), temperature (4–55 ˚C), fermentation time (24–96 h), inoculum volume (2-8% v/v), shaker speed (120-180 rpm) and non aqueous systems on the production of ALP was studied. The growth was measured as optical density at 600 nm. 1 ml of the fermented broth was centrifuged at 7000 x g at 4 °C for 15 min and cell free supernatant was used for determination of ALP productivity.

Analysis of ALP

ALP activity was measured spectrophotometrically by determining the release of *p*-nitrophenol (*p*-NP) from *p*-nitrophenyl phosphate disodium salt (*p*-NPP) at 400 nm (Garen and Levinthal, 1960; Zappa, 2001; Robert, 2003). 100 µl cell free supernatant was added to 1000 µl of *p*-NPP solution (1.35 mM in 50 mM Tris-HCl buffer at pH 9.0) and the mixture was incubated at 35 ˚C for 10 min. One unit of enzyme activity is the amount of the ALP catalyzing the liberation of 1µmol of *p*-NP per min.

Results and discussions

The strain

On the basis of 16s rDNA partial sequencing,the bacterial isolate FPB17 was identified as *Bacillus flexus* and the sequence has been submitted to NCBI GenBank with accession number as JN415115.

Effect of initial pH and temperature

Results obtained of the effect of initial pH and temperature on ALP production. It was observed that optimum pH for the growth of the bacterium was 10.0, while that for ALP production, it was 9.0. ALP production declined below and above the pH optima (Figs. 1 and 2). ALP production under alkaliphilic pH range have been reported in *E. coli* (pH 8.3) by Danielle and Raymond, 1984; in *Penicillium expansum* (pH 9.5) by Dahot *et al*., 1986; and in *Neurospora crassa* (pH 9.5-10.5) by Morales (2000). The optimum ALP enzyme production was observed at 35 ºC and there was complete inhibition of productivity at 55 ºC or below 15 ºC (Fig. 2). Slightest deviation in incubation temperature results in major loss of enzyme production. Temperatures in the range of 37 °C to 40 °C have been reported as optimum for ALP production in *E. coli* by Danielle and Raymond, 1984 and in *Penicillium expansum* by Dahot *et al*. (1986).

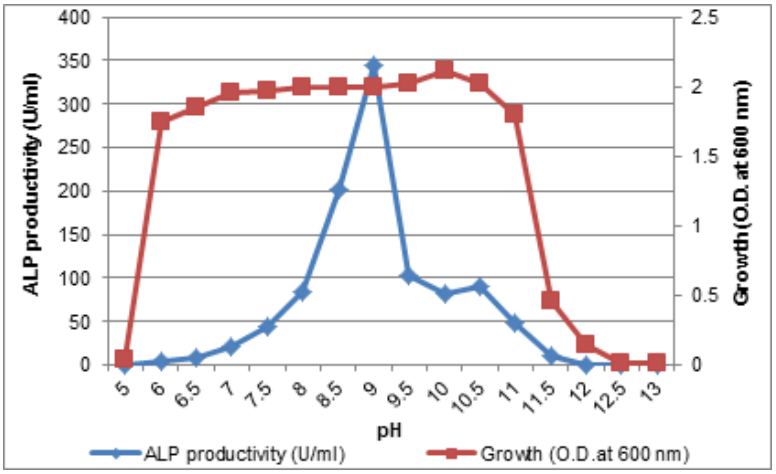

Fig. 1. Effect of pH on the growth and ALP production by *Bacillus flexus* FPB17

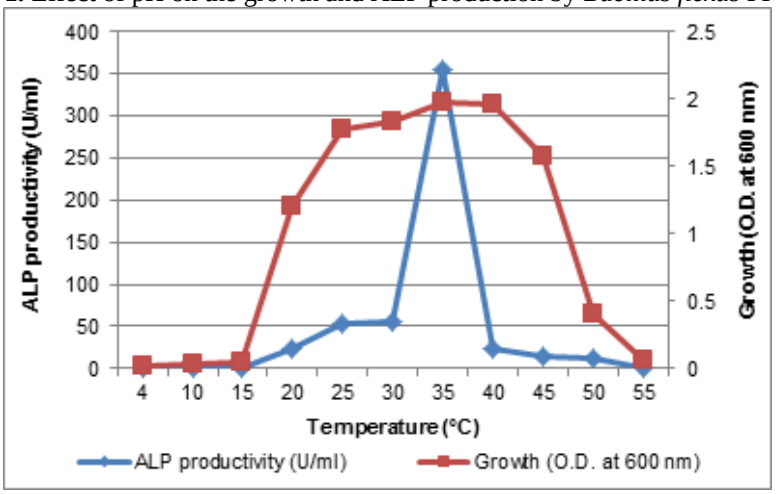

Fig. 2. Effect of incubation temperature on the growth and ALP production by *Bacillus flexus* FPB17

Effect of incubation period, inoculum volume and shaker speed

The observations of the effect of incubation period, inoculum volume and shaker speed on ALP production were depicted in Fig. 3 to 5. The maximum production of enzyme occurred in its stationary phase, after 48 h of fermentation (Fig. 3). Further incubation resulted in decline of enzyme level. 2% v/v inoculum volume was optimum for production of ALP, additional inoculum volume resulted in higher growth but lower ALP production (Fig. 4). Agitation rate of 140 rpm proved optimum for ALP production, increase in agitation rate resulted in increased biomass but lower ALP levels (Fig. 5). In all cases, the increase in growth was found to be associated with decline in ALP productivity. Therefore ALP is not a growth associated metabolite. It is likely

that substrates get diverted in to the cell mass build up and are not available to the ALP biosynthesis route. *Bacillus flexus* FPB17 being aerobic, required aeration but is not high oxygen requiring.

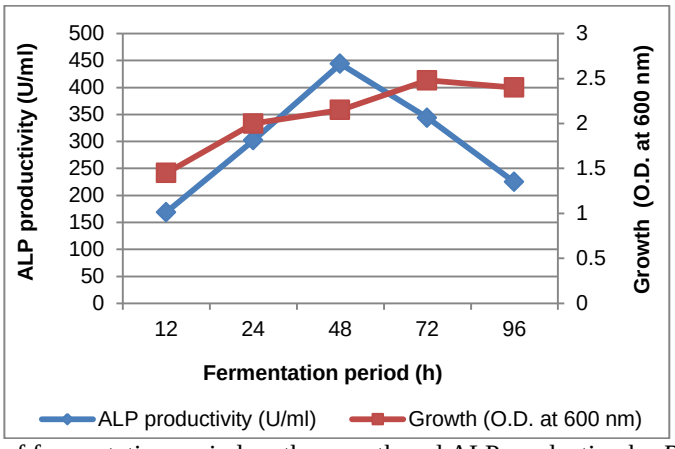

Fig. 3. Effect of fermentation period on the growth and ALP production by *Bacillus flexus* FPB17

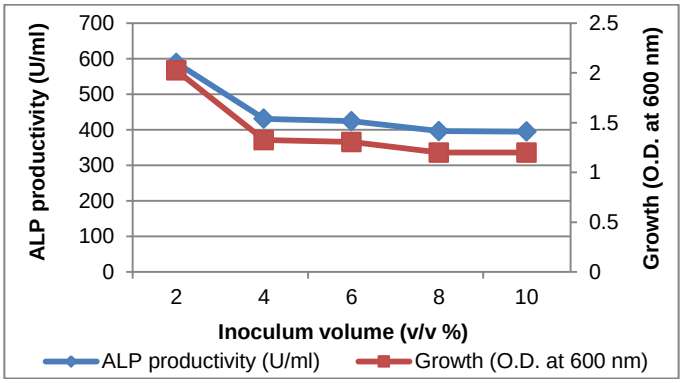

Fig. 4. Effect of inoculum volume on the growth and ALP production by *Bacillus flexus* FPB17

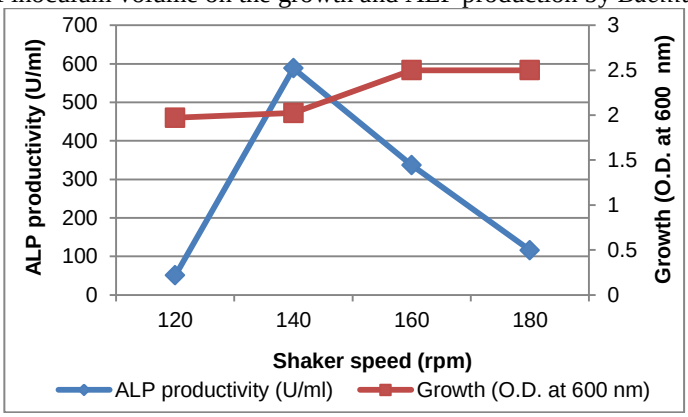

Fig. 5. Effect of shaker speed on the growth and ALP production by *Bacillus flexus* FPB17.

Effect of non aqueous fermentation system

The ALP productivity was repressed in non aqueous systems even in the presence of lowest concentration of solvents tested (10% v/v concentration of benzene, acetone, methanol, 2-propanol, ethanol and hexane) in the production medium. Maximum inhibition of enzyme productivity was observed with 2-propanol and ethanol (Fig. 6). Hydrolases are known to be very stable in non-aqueous environments, this being particularly true for esterases, lipases and proteases produced by strains of *Pseudomonas* and *Bacillus*, particularly in extremophilic strains. However, it is very well documented that enzymes are denatured or highly inactivated in the presence of organic solvents, and specific catalytic activities of enzymes that are stable in non-aqueous environments are generally lower than those in aqueous systems (Torres and Castro, 2004).

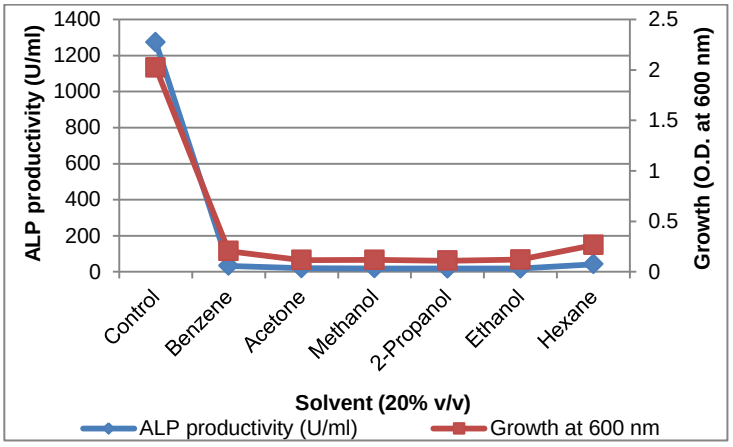

Fig. 6. Effect of non aqueous fermentation system on the growth and ALP production by *Bacillus flexus* FPB17

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