Journal of Agricultural Technology 2013 Vol. 9(1): 81-93
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Optimization of metal salt requirement for production of alkaline phosphatase by a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 using response surface methodology

Patel Falguni R.* **and M.C. Sharma**

Department of Biotechnology, Kadi Sarva Vishwavidyalaya, Sector-23, Gandhinagar-382023, India

Patel Falguni R. and M.C. Sharma (2013) Optimization of metal salt requirement for production of alkaline phosphatase by a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 using response surface methodology. Journal of Agricultural Technology 9(1):81-93.

Optimization of metal salts concentration (screened by Placket–Burman design) was carried out for alkaline phosphatase production by a halotolerant facultative alkaliphile *Bacillus flexus* FPB17 through Response surface methodology. Eleven metal salts including NaCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, KCl, $ZnCl_2$, NH_4Cl and $Pb(NO_3)_2$ were screened using Placket–Burman design criterion. Four salts *viz.* $CaCl_2$, $MgCl_2$, KCl and NaCl showed significant effect ($p < 0.05$) on alkaline phosphatase production. The metal salt concentrations were optimized by Central Composite Design, the most suitable metal salt composition being $CaCl_2$ – 0.05 g%; $MgCl_2$ – 0.05 g%; KCl – 0.01 g% and NaCl – 0.5 g%. At these optimum levels of metal salts, 239.58 U/ml alkaline phosphatase was produced. Selection and optimization of metal salts by Response surface methodology resulted in 1.84-fold increased yields of alkaline phosphatase.

Key words: *Bacillus flexus* FPB17, halotolerant facultative alkaliphile, Placket–Burman design, Response surface methodology

Introduction

Alkaline phosphatases (ALPs; E.C.3.1.3.1) are nonspecific, phosphomonoesterases, exhibited by a wide variety of organisms (Simao *et al.*, 2007; Barbosa jr. *et al.*, 2008). These hydrolyze a wide variety of phosphate esters and are crucial for survival of organisms to provide inorganic phosphate (Pi) nutrition for synthesis of nucleic acids, phosphorylated sugars and proteins, *etc.* (De Prada *et al.*, 1996; Zappa *et al.*, 2001).

ALPs have been utilized in molecular biology primarily for dephosphorylation of 5'-phosphorylated DNA or RNA ends and the construction of recombinant DNA molecules. The other applications of ALP

* Corresponding author: Patel Falguni R; e-mail: fal2910@yahoo.co.in

include diagnostics by immunoassays (EIA), chemiluminescent immunoassays, and ALP-labeled nucleic probes / other probes for detection of hepatobiliary disease & several skeletal dysfunctions, inflammatory bowel disease therapy of lipopolysaccharide mediated diseases and studies of phosphoproteins (Deftos, 1991; Christenson, 1997; Beumer *et al.*, 2003). In the dairy industry, ALP activity is an indicator for adequate pasteurization of milk (Fenoll *et al.*, 2002) and also used for the analysis of tannins in grapes and red wines (Adams and Harbertson, 1999). Chen *et al.*, 1996 described the use of ALP in chemiluminescence-based biosensors for the determination of pesticides or heavy metals in water, particularly for organophosphorus pesticide. The use of ALP was also done in optical (Durrieu and Tran-Minh, 2002), amperometric (Mazzei *et al.*, 2004), fluorimetric (Sanchez *et al.*, 2004), colorimetric biosensors (Goh *et al.*, 2005). ALP finds applications in efficient removal of phosphorus during wastewater treatment. ALPs are by far the most studied type in aquatic ecosystems and sewage sludge treatments (Jansson *et al.*, 1988; Albiach *et al.*, 2000; Anupama *et al.*, 2008). Successful bioprecipitation of metals such as uranium and cadmium has been done through the phosphatases from naturally occurring bacteria (Macaskie *et al.*, 2000).

ALPs are metalloenzymes (Posen, 1967), and they are all inhibited by metal ion chelators such as EDTA (Aehle, 2007). They are classically considered to be Zn^{+2}- and Mg^{+2}- dependent enzymes, especially *E. coli* and mammalian ALPs (Kim and Wyckoff, 1989). However, activation following addition of Mn^{+2}, Co^{+2}, or other metal ions has already been observed among other ALPs (Yamashita *et al.*, 1990; Mori *et al.*, 1999). ALP from psychrophilic bacterium *Bacillus* sp. P9 also required the metal ions Mg^{+2}, Ca^{+2} and Zn^{+2} as activators (Dhaked *et al.*, 2005).

Effect of metal ions on the activity and kinetic properties of phosphatase have been reported by Flint and Hopton, 1977; Huang and Shindo, 2000. Banik and Pandey, 2009 checked the effect of different eight metal salts $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $MgSO_4$, $MnSO_4$, NaCl and $Pb(NO_3)_2$ on ALP production by *Bacillus licheniformis* by using Response surface methodology (RSM). RSM is advantageous over conventional methods available and it includes less experiment numbers, its suitability for multiple factor experiments and search for common relationship between various factors towards finding the most suitable production conditions for the bioprocess and forecast response (Kaur and Satyanarayana, 2005).

The work described in this article deals with optimization of salts concentration for ALP production by *Bacillus flexus* FPB17. In this paper we had studied the effect of different eleven metal salts *viz.* NaCl, $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, KCl, $ZnCl_2$, NH_4Cl and $Pb(NO_3)_2$ for secretion

and solubilization of ALP using Response surface methodology (RSM). The metal salts having significant effect on ALP production were selected by Placket–Burman Design and Central Composite Design (CCD) was used to optimize the concentration of the selected metal salts for the production of ALP.

Materials and methods

Culture and production medium conditions

Bacillus flexus FPB17, the isolate from the sediment sample of an alkaline lake located in Bhilot village of Patan district, Gujarat, identified on the basis of 16S rRNA sequencing (sequence accession number JN415115 of NCBI GenBank), was maintained on Nutrient agar slants containing 1% peptone, 1% meat extract, 0.5% NaCl and 2% agar. Inoculum preparation and ALP production were studied 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, 140 rpm on an orbital shaker.

Analysis of ALP

ALP activity was measured spectrophotometrically in the form of Unit/ml (U/ml) 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 *et al.,* 2001; Robert and Evan, 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.

Optimization of ALP production by Bacillus flexus FPB17

The different metal salts and their levels were selected on the basis of experiments carried out by Banik and Pandey, 2009 who reported the selection and optimization of metal salts for ALP production by *Bacillus licheniformis* using RSM.

Effect of Salts on ALP production

The concentration of NaCl in the range of 0-12% in N. broth was studied arbitrarily first. The selection of other significant metal salts with NaCl for production of ALP was done by using RSM, a statistics based design which was applied in two stages, first to identify the significant salts for production of ALP by using Placket–Burman Design criterion and later the significant salts deduced from Placket–Burman Design were optimized by using CCD. The experimental design and statistical analysis of the data were done by using Design Expert software package (version-8.0.6.1), Stat-Ease Inc., Minneapolis (MN).

Placket–Burman Design

Each variable was tested at two levels namely a high level denoted by (+1) and a low level denoted by (-1) as listed in Table 1. Eleven variables were screened by conducting twelve experiments and the experimental design was given in Table 2. All experiments were conducted in triplicate and the average value of ALP yield was used for statistical analysis. The variables, which were significant at 5% level ($P < 0.05$) from the regression analysis were considered to have greater impact on ALP production and were further optimized using CCD.

Table 1. Level of metal salts studied for the production of ALP by FPB17 using Placket– Burman design criterion

Factor	Name	Units	Minimum (-1)	Maximum (+1)
A	NaCl	g%	0.10	0.50
B	$MgCl_2$	g%	0.01	0.05
C	$MnCl_2$	g%	0.01	0.05
D	$ZnCl_2$	g%	0.01	0.05
E	$CoCl_2$	g%	0.01	0.05
F	$CaCl_2$	g%	0.01	0.05
G	NH_4Cl	g%	0.01	0.05
H	KCl	g%	0.01	0.05
J	$CuCl_2$	g%	0.01	0.05
K	$FeCl_3$	g%	0.01	0.05
L	$Pb(NO_3)_2$	g%	0.01	0.05

Table 2. Placket–Burman design for study of effect of metal salts on the production of ALP by FPB17

Run order	A:	B:	C:	D:	E:	F:	G:	H:	J:	K:	L:
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
2	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
3	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
4	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
5	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
6	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
7	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
8	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
9	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
10	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
12	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1

where A = NaCl, B = $MgCl_2$, C = $MnCl_2$, D = $ZnCl_2$, E = $CoCl_2$, F = $CaCl_2$, G = NH_4Cl, H = KCl, J = $CuCl_2$, K = $FeCl_3$, L = $Pb(NO_3)_2$

Central Composite Design (CCD)

CCD was applied to determine the optimum concentration of three most significant metal salts other than NaCl screened through Placket–Burman Design criterion. The design with six start points and six replicates at the central point, resulting in 20 experiments was generated by Design Expert, Version 8.0.6.1, as mentioned earlier. The levels of factors used for experimental design are given in Table 1 and the coded variables are calculated according to the equation 1 and the behavior of the system was explained by the following second order polynomial equation 2. Both the equations are given below:

( )

∑ ∑ ∑

Where Y is the predicted response, X_i, X_j are input variables which influence the response variable Y; b_0 is the offset term; b_i is the i^{th} linear coefficient; b_{ii} is the i^{th} quadratic coefficient and b_{ij} is the ij^{th} interaction coefficient. Analysis of variance (ANOVA), regression analysis were done and contour plots were drawn by using Design Expert.

Results and discussions

Effect of Sodium Chloride concentration on ALP production by Bacillus flexus

Varying concentrations of NaCl in the range of 0-12 g% in N. broth were taken arbitrarily first to analyze their effect on extracellular ALP production. The maximum enzyme activity was 104.16 U/ml, obtained with 0.5 g% NaCl concentration. Further increase in NaCl inhibited the ALP production (Fig. 1). *Bacillus flexus* is reported to be generally halotolerant upto 12% NaCl concentration (Priest *et al.*, 1988), but this FPB17 strain was an exception to this character as the growth drops drastically beyond 5% NaCl level indicating that FPB17 is halotolerant rather than a halophile.

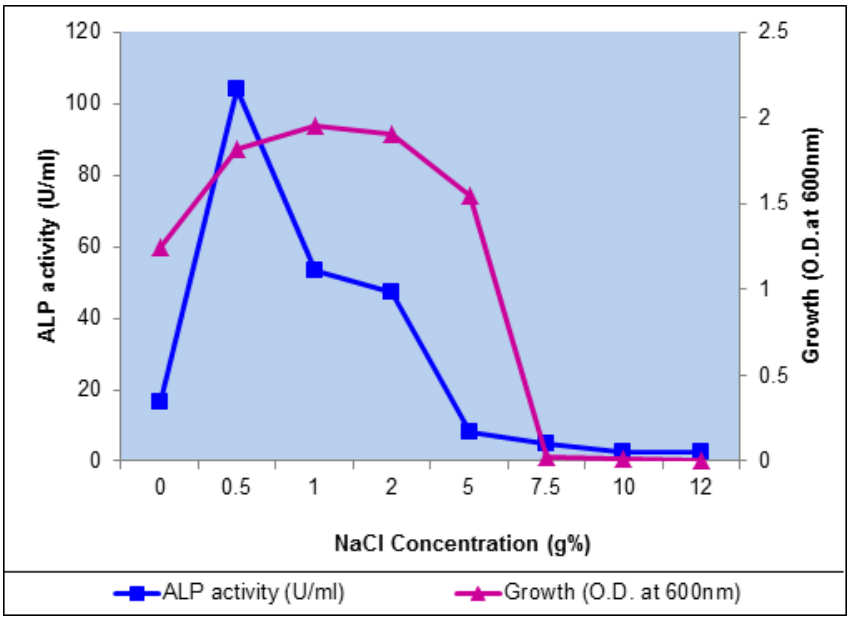

Fig. 1. Effect of NaCl concentration on ALP production by *Bacillus flexus* FPB17.

Screening for significant metal salts using Placket–Burman Design criterion

The Placket–Burman Design based experiment indicated significant stimulatory effect of NaCl, $MgCl_2$, $CaCl_2$ and KCl on ALP production by *Bacillus flexus* FPB17 as exhibited by Fig. 2.

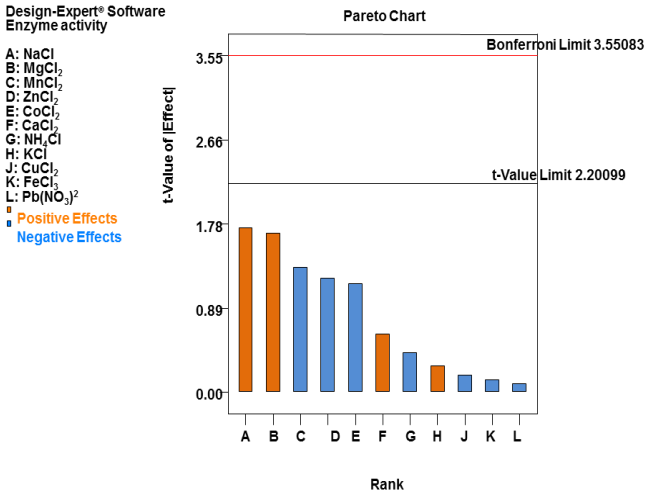

Fig. 2. Pareto chart of effect of metal salts for the production of ALP by *Bacillus flexus* FPB17 using Placket-Burman design criterion.

Optimization of metal salts concentration using CCD for ALP production by Bacillus flexus FPB17

Twenty experiments (and one control) carried out according to the CCD as shown in Table 3 indicate major role of $MgCl_2$-$CaCl_2$ interaction. Multiple regression analysis of the application data indicates following second order polynomial equation explaining the ALP production by *Bacillus flexus* FPB17:

$$Y = 114.44 + 20.93A + 3.96B - 28.11C + 17.64AB - 39.36AC + 5.19BC + 1.38A^2 + 0.81B^2 + 5.16C^2$$

Where Y is the predicted response variable, ALP activity (U/ml) and A, B and C are the values of independent variables $MgCl_2$, $CaCl_2$ and KCl, respectively.

Regression analysis of the experimental data showed that $MgCl_2$, $CaCl_2$ and KCl had positive effect on ALP production ($P < 0.05$). Amongst the three metal salts $MgCl_2$ and $CaCl_2$ had highest impact on ALP production as shown by highest linear coefficient followed by KCl. These salts also showed significant quadratic effect on ALP production. The interactions between $MgCl_2$-$CaCl_2$ and $MgCl_2$-KCl were significant as shown by low P values (<0.05) for interactive terms. But the interaction between $CaCl_2$-KCl was found to be insignificant as the p values are above 0.05 (Table 4). Analysis of variance for the ALP production obtained from this design is presented in Table 5. ANOVA gave the value of the model and explains that this model adequately

fits the variation observed in ALP production with the designed salt levels. The closer the value of R (multiple correlation coefficient) to 1, better is the correlation between the observed and predicted values.

Table 3. Optimization of significant metal salts for ALP production by *Bacillus flexus* FPB17 using Central Composite Design criterion

Run order	A: $MgCl_2$ g%	B: $CaCl_2$ g%	C: KCl g%	ALP activity (U/ml) Predicted	Experimental	Residuals
1	0.06	0.03	0.03	153.55	147.08	-6.47
2	0.03	0.03	0.03	114.44	114.69	0.25
3	0.03	0.03	0.03	114.44	113.86	-0.58
4	0.03	0.03	0.06	81.78	79.97	-1.81
5	0.05	0.05	0.01	226.59	239.58	12.99
6	0.03	0.03	0.03	114.44	114.69	0.25
7	0.01	0.05	0.01	70.75	71.45	0.70
8	0.03	0.06	0.03	123.38	111.91	-11.47
9	0.00	0.03	0.03	89.26	89.56	0.30
10	0.05	0.01	0.01	193.77	189.69	-4.08
11	0.01	0.05	0.05	103.63	112.16	8.53
12	0.03	0.03	0.03	114.44	114.69	0.25
13	0.03	0.03	0.03	114.44	114.75	0.31
14	0.01	0.01	0.05	120.61	112.06	-8.55
15	0.03	0.03	0.00	160.46	153.17	-7.29
16	0.03	0.00	0.03	110.62	119.06	8.44
17	0.03	0.03	0.03	114.44	114.48	0.04
18	0.05	0.05	0.05	102.04	103.92	1.88
19	0.01	0.01	0.01	108.48	111.05	2.57
20	0.05	0.01	0.05	48.47	52.21	3.74
Control	0.00	0.00	0.00	–	129.86	–

Table 4. Regression analysis of Central Composite Design criterion based data for ALP production by *Bacillus flexus* FPB17

Factor	**Coefficient**	**Standard Error of Coefficient**	**T Value**	**p-value**
Constant	114.44	3.20	35.76	0.00
$MgCl_2$	20.93	2.33	8.98	0.00
$CaCl_2$	3.96	2.33	1.70	0.12
KCl	-28.11	2.33	-12.06	0.00
$MgCl_2 * CaCl_2$	17.64	2.87	6.15	0.00
$MgCl_2 * KCl$	-39.36	2.87	-13.71	0.00
$CaCl_2 * KCl$	5.19	2.87	1.81	0.10
$MgCl_2 * MgCl_2$	1.38	2.48	0.56	0.59
$CaCl_2 * CaCl_2$	0.81	2.48	0.33	0.75
$KCl * KCl$	5.16	2.48	2.08	0.06

$R^2 = 97.8\%$

Table 5. Analysis of variance for ALP production by *Bacillus flexus* FPB17 using Central Composite Design criterion

Source	Sum of Squares	Degree of freedom (D_f)	Mean Square	F Value	p-value
Model	30503.57	9	3389.29	51.54	0.00
$MgCl_2$	5297.23	1	5297.23	80.56	0.00
$CaCl_2$	189.75	1	189.75	2.89	0.12
KCl	9555.83	1	9555.83	145.32	0.00
$MgCl_2 * CaCl_2$	2488.65	1	2488.65	37.85	0.00
$MgCl_2 * KCl$	12392.10	1	12392.10	188.46	0.00
$CaCl_2 * KCl$	215.49	1	215.49	3.28	0.10
$MgCl_2 * MgCl_2$	20.52	1	20.52	0.31	0.59
$CaCl_2 * CaCl_2$	6.95	1	6.95	0.11	0.75
KCl * KCl	285.52	1	285.52	4.34	0.06
Residual	657.55	10	65.76	–	–
Lack of Fit	656.97	5	131.39	–	–
Pure Error	0.58	5	0.12	–	–
Cor Total	31161.12	19	–	–	–

In the present study the value of R (0.978) revealed that the model could explain up to 97.8% variation of ALP production. The p value for the model (0.00) indicated that the experimental data obtained fitted well with the model and explained the effect of $MgCl_2$, $CaCl_2$ and KCl on ALP production by *Bacillus flexus*. The 2D contour plots of ALP production for each pair of salt concentration by keeping the other one metal salt constant are shown in Fig. 3, 4 and 5. The 2D contour plots are the graphical representation of the regression equation. The main goal of response surface is to efficiently hunt for the optimum values of the variables such that the response is maximized. The optimal combination of the metal salts of media for ALP production as obtained from the contour plots are as follows: $CaCl_2$ – 0.05 g%; $MgCl_2$ – 0.05 g%; KCl – 0.01 g% and NaCl – 0.5 g%. At these optimum levels of metal salts, 1.84-fold increase in the ALP yield amounting to 239.58 U/ml was achieved with RSM.

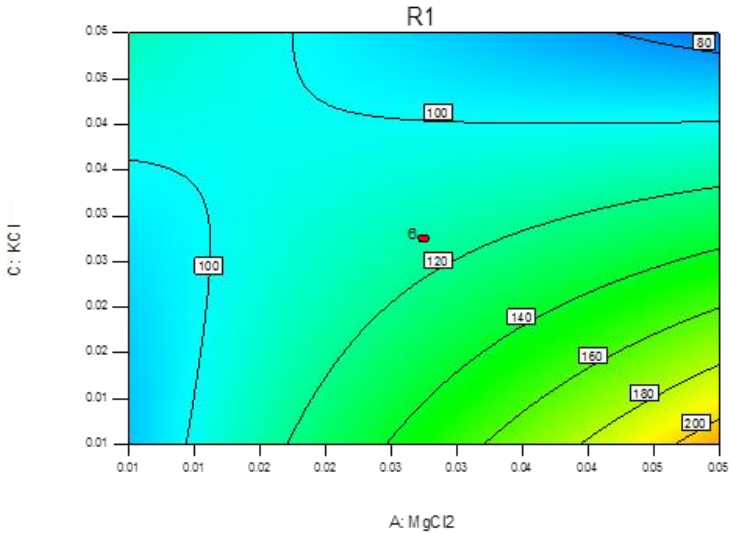

Fig. 3. Contour plot of effect of $MgCl_2$ and KCl on ALP production.

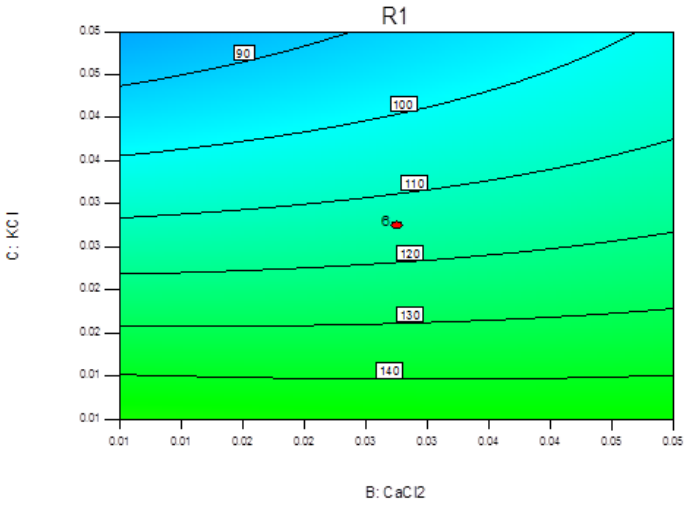

Fig. 4. Contour plot of effect of $CaCl_2$ and KCl on ALP production.

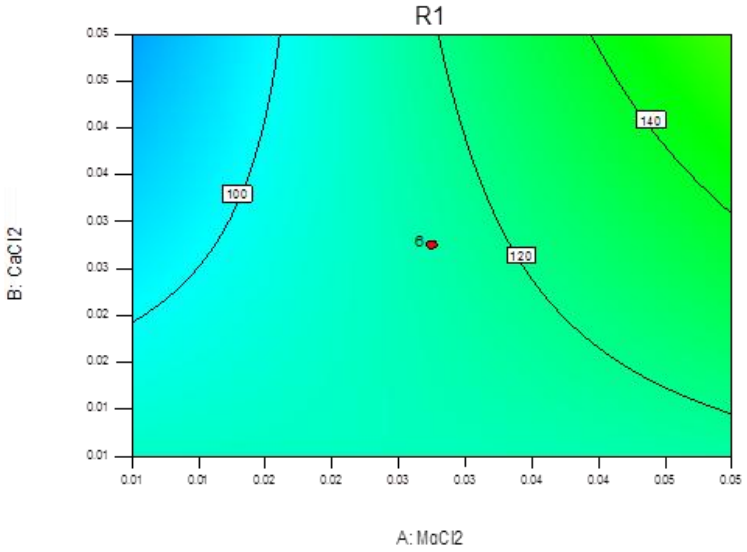

Fig. 5. Contour plot of effect of $MgCl_2$ and $CaCl_2$ on ALP production.

References

Adams, D.O. and Harbertson, J.F. (1999). Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. American Journal of Enology and Viticulture 50:364-384.

Aehle, W. (2007). Enzymes in industry: Production and applications, 3rd edition, Wiley VCH Verlag Gmbh & Co. KGaA, Weinheim.

Albiach, R., Canet, R., Pomares, F. and Ingelmo, F. (2000). Microbial biomass content and enzymatic activities after the application of organic amendments to a horticultural soil. Bioresource Technology 75:43–48.

Anupama, V.N., Amrutha, P.N., Chitra, G.S. and Krishnakumar, B. (2008). Phosphatase activity in anaerobic bioreactors for wastewater treatment. Water Research 42:2796-2802.

Banik, R.M. and Pandey, S.K. (2009). Selection of metal salts for alkaline phosphatase production using response surface methodology. Food Research International 42:470–475.

Barbosa jr., A., Guimaraes, L.H.S, Terenzi, H.F., Jorge, J.A., Leone F.A. and Polizeli, M.L.T.M. (2008). Purification and biochemical characterization of thermostable alkaline phosphatase produced by *Rhizopus microspores* var *rhizopodiformis*. Folia Microbiologica 53:509-519.

Beumer, C., Wulferink, M., Raaben, W., Fiechter, D.L., Brands, R. and Seinen, W. (2003). Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. Journal of Pharmacology and Experimental Therapeutics 307:737-744.

Chen, Z., Kaplan, D.L., Gao, H., Kumar, J., Marx, K.A. and Tripathy, S.K. (1996). Molecular assembly of multilayer enzyme: toward the development of chemiluminescence-based fiber optic biosensor. Material Science and Engineering 4:155–159.

Christenson, R.H. (1997). Biochemical markers of bone metabolism: an overview. Clinical Biochemistry 30:573-593.

De Prada, P., Loveland-Curtze, J. and Brenchley, J.E. (1996). Production of two extracellular alkaline phosphatases by a psychrophilic *Arthrobacter* strain. Applied and Environmental Microbiology 62:3732-3738.

Deftos, L.J. (1991). Bone protein and peptide assays in the diagnosis and management of skeletal disease. Clinical Chemistry 37:1143-1148.

Dhaked, R.K., Alam, S.I., Dixit, A. and Singh, L. (2005). Purification and characterization of thermolabile alkaline phosphatase from an antarctic psychrotolerant *Bacillus* sp. P9. Enzyme and Microbial Technology 36:855-861.

Durrieu, C. and Tran-Minh, C. (2002). Optical algal biosensor using alkaline phosphatase for the determination of heavy metals. Ecotoxicology and Environmental Safety 51:206–209.

Fenoll, J., Jourquin, G. and Kauffmann, J.M. (2002). Flourimetric determination of alkaline phosphatase in solid and fluid dairy products. Talanta 56:1021-1026.

Flint, K.P. and Hopton, J.W. (1977). Substrate specificity and ion inhibition of bacterial and particle associated alkaline phosphatases of waters and sewage sludges. European Journal of Applied Microbiology 4:195-204.

Garen, A. and Levinthal, C. (1960). A fine structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli* I. Purification and characterization of alkaline phosphatase. Biochimica et Biophysica Acta 38:470-483.

Goh, C.T., Ahmed, M. and Lee, Y.H. (2005). Fabrication of alkaline phosphatase biosensor for Hg/sup 2+/determination. Proceeding of 2005 Asian conference on sensors and the international conference on new techniques in pharmaceutical and biomedical research, September 5-7, Kuala Lumpur, Malaysia, pp. 99-102.

Huang, Q. and Shindo, H. (2000). Effects of copper on the activity and kinetics of free and immobilized acid phosphatase. Soil Biology and Biochemistry 32:1885–1892.

Jansson, M., Olsson, H. and Pettersson, K. (1988). Phosphatases: origin, characteristics and function in lakes. Hydrobiologia 170:157–175.

Kaur, P. and Satyanarayana, T. (2005). Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: Optimization using statistical tools. Process Biochemistry 40:3095-3102.

Kim, E.E. and Wyckoff, H.W. (1989). Structure of alkaline phosphatase. Clinica Chimica Acta 186:175-188.

Macaskie, L.E., Bonthrone, K.M., Yong, P. and Goddard, D.T. (2000). Enzymically mediated bioprecipitation of uranium by a *Citrobacter* sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. Microbiology 146:1855–1867.

Mazzei, F., Botr`e, F., Montilla, S., Pilloton, R., Podest`a, E. and Botr`e, C. (2004). Alkaline phosphatase inhibition based electrochemical sensors for the detection of pesticides. Journal of Electroanalytical Chemistry 574:95–100.

Mori, S., Okamoto, M., Nishibori, M., Ichimura, M., Sakiyama, J. and Endo, H. (1999). Purification and characterization of alkaline phosphatase from *Bacillus stearothermophilus*. Biotechnology and Applied Biochemistry 29:235-239.

Posen, S. (1967). Alkaline phosphatase. Annals of Internal Medicine 67:183–203.

Priest, F.G., Goodfellow, M. and Todd, C. (1988). A numerical classification of the genus *Bacillus.* Journal of General Microbiology 134:1847-1882.

Robert, R.B. and Evan, R.K. (2003). Characterization of a monomeric *E. coli* alkaline phosphatase formed upon a single amino acid substitution. Journal of Biological Chemistry 278:23497-23501.

Sanchez, F.G., Diaz, A.N., Peinado, M.C.R. and Belledone, C. (2003). Free and sol-gel immobilized alkaline phosphatase-based biosensor for the determination of pesticides and inorganic compounds. Analytica Chimica Acta 484: pp. 45.

Simao, A.M.S., Beloti, M.M., Rosa, A.L., Oliveria, P.T., Granjeiro, J.M., Pizauro, J.M. and Ciancaglini, P. (2007). Culture of osteogenic cells from human alveolar bone: A useful source of alkaline phosphatase. Cell Biology International 31:1405-1413.

Yamashita, Y., Toyoshima, K., Yamazaki, M., Hanada, N. and Takehara, T. (1990). Purification and characterization of alkaline phosphatase of *Bacteroides gingivalis* 381. Infection and Immunity 58:2882-2887.

Zappa, S., Rolland, J.L., Flament, D., Gueguen, Y., Boudrant, J. and Dietrich, J. (2001). Characterization of a highly thermostable alkaline phosphatase from the Euryarchaeon *Pyrococcus abyssi.* Applied and Environmental Microbiology 67:4504-4511.

(Received 27 July 2012; accepted 30 December 2012)