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Selection and optimization of nutritional constituents for enhanced production of alkaline phosphatase by *Bacillus licheniformis* MTCC 1483

S.K. Pandey and R.M. Banik*

School of Biochemical Engineering, Institute of Technology, Banaras Hindu University, Varanasi-221005, India

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A study was made for selection and optimization of nutritional constituents including macro and micronutrients to enhance the alkaline phosphatase production. Among various carbon sources, nitrogen sources and metal salts used, glucose, peptone, yeast extract, $MgSO_4$, $CaCl_2$ and NaCl were found to be the most appropriate nutritional constituents to enhance the alkaline phosphatase production. The concentration of glucose, peptone and yeast extract was optimized using central composite design. The P-value of the coefficient for linear and interactive effect of glucose, peptone and yeast extract were found to be less than 0.05 (<0.05). The experimental values` are in good agreement with predicted values and the correlation coefficients (R^2) were found to be 0.932 (93.2%). Several metal salts were applied with optimized concentration of glucose, peptone and yeast extract and observed that $MgSO_4$, $CaCl_2$, and NaCl are responsible to increase the alkaline phosphatase production. The optimal combination of the nutritional constituents in 100 ml media solution was glucose-2.39%, peptone-1.35%, yeast extract-0.15% $MgSO_4$-0.08%, $CaCl_2$-0.1% and NaCl-0.5%. At these optimum levels of nutrients, alkaline phosphatase production of 2670.67 U/ml was obtained.

Keywords: Alkaline phosphatase, *Bacillus licheniformis,* Production, Optimization, Central Composite Design

Introduction

Alkaline phosphatases [orthophosphate monoester phosphohydrolases EC 3.1.3.1] are metalloenzymes, nonspecific, phosphomonoesterases that exist in various organisms from bacteria to mammals (Oh *et al*., 2007; Simao *et al*., 2007; Junior *et al*., 2008; Huang *et al*., 2009; Sasajima *et al*., 2010). Alkaline phosphatase hydrolyzes a wide variety of phosphate esters and is classified as alkaline phosphatase according to its optimum pH ranging from 7.5 to 11.0

*Corresponding author: R.M. Banik; e-mail: rmbanik@gmail.com, sanjeevbiochem11@gmail.com

(Dhaked *et al*., 2005; Gong *et al*., 2005; Lu *et al*., 2010). In a wide variety of organisms, alkaline phosphatase plays a vital role in phosphate transportation and metabolism and is a most crucial enzyme for the survival of organisms under phosphate starvation (Fernandes *et al*., 2008). The enzyme has been widely used in the diagnostics, immunology and molecular biology as biochemical markers in quantitative measurements of disease, linked enzymes in ELISA (Chen *et al*., 2006; Sun *et al*., 2007; Muginova *et al*., 2007; Baranov *et al*., 2008) and used in non radioactive detection techniques, probing, blotting and sequencing systems (Nilgiriwala *et al*., 2008; Sasajima *et al*., 2010).

Because of the important principle and practical values of the enzyme, more and more research interests have been attracted in the field. So far, the most commonly used alkaline phosphatases are *Escherichia coli* alkaline phosphatase and calf intestine alkaline phosphatase (Lorenz and Schroder, 2001; Suzuki *et al*., 2005), but their inherently low thermal resistance and shelf lives have restricted their further applications under some special circumstances, for instance, under high temperature. Therefore, thermostable alkaline phosphatase has attracted many attentions because it has the advantage of being used under some extreme conditions (Zappa *et al*., 2001; Wojciechowski and Kantrowitz, 2002). Alkaline phosphatase from various microorganisms belonging to *Bacillus sp.* is thermostable which are preferred currently for genetic engineering, biotechnological and industrial applications (Mikhaleva *et al*., 1995; Badyakina *et al*., 2003). The present study is totally based on the selection and optimization of nutritional constituents for high yield of thermostable *Bacillus licheniformis* alkaline phosphatase (Pandey and Banik, 2011). Carbon and nitrogen are essential components for microbial anabolic system therefore the proper concentration of carbon and nitrogen is required for microbial growth and enzyme production.The metabolic process of any microorganism is totally depend upon the carbon and inorganic elements (nutrients) such as nitrogen, phosphorus, sulfur, potassium, calcium, and magnesium as an energy source to synthesize new cellular materials (Goldman and Mark, 2000). Organic carbons are the common substrates *i.e*. usually referred to heterotrophic microorganisms for the main energy sources. Nitrogen is required for protein synthesis which is responsible for about 12-16% of dry cell mass (Quagliano and Miyazaki, 1997). Metal cations represent the exogenous folding effectors in *Bacilli*. Membrane of *Bacilli is* surrounded by moderately thick cell wall (from 10 to 50 nm) of heteropolymeric matrix that consists of peptidoglycan and teichoic acids (Yurchenko*et al*., 2003). Carboxyl groups and phosphate groups of peptidoglycan and teichoic acid determine anionic nature of *Bacilli* cell wall, which can bind positively charged cations, Mg^{2+}, Ca^{2+}, Fe^{3+}, etc. (Guimaraes *et al*., 2001; Yurchenko *et al*., 2003). The

alkaline phosphatase from *B. licheniformis* exists as a membrane associated enzyme (Hulett and Campbell, 1971; Spencer *et al*., 1982) and as a soluble secreted enzyme (Glynn*et al*., 1977; Spencer *et al*., 1981). The membrane associated alkaline phosphatase has been solubilized with Mg^{2+} (Schaffel and Hulett, 1978). In complex medium the soluble enzyme is not secreted into the medium but is released by solubilizing with cationic metal salts. The secretion and solubilization of membrane associated alkaline phosphatase is stimulated by various cationic metal salts such as Zn^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+} (Yamane and Maruo, 1978; Kelly *et al*., 1984; Sugahara *et al*., 1991; Choi *et al*., 2000). In biotechnology, the one at a time approach is the most frequently used operation to optimize all process parameters and nutrient concentration for maximum cell density, high yields of the desired metabolic product, or enzyme levels in a microbial system. This approach is not only time consuming, but ignores the combined interactions among various physicochemical parameters. On the contrary, the response surface methodology (RSM), which includes factorial design and regression analysis, helps in evaluating the effective factors and building models to study interaction and select optimum conditions of variables for a desirable response (Ya-Hong *et al*., 2004; Banik *et al*., 2007). Recently, a number of statistical experimental designs with response surface methodology have been employed for optimizing enzyme production from microorganism (Santhiagu and Banik, 2008; Banik and Pandey, 2009; Pandey and Banik, 2010). The work described in this article deals with selection of macronutrients (carbon and nitrogen) and micronutrients (metal salts) for high yield of alkaline phosphatase. Optimization of significant carbon and nitrogen concentration were carried out using central composite design. Finally, several metal salts were used with optimized concentration of carbon and nitrogen source to get high yield of alkaline phosphatase.

Materials and methods

Organism and chemicals

Bacillus licheniformis (MTCC 1483) was collected from Institute of Microbial Technology, Chandigarh, India. The enzyme substrate, sodium salt of p-nitro phenyl phosphate (PNPP), Tris-HCl buffer, and carbon sources (glucose, lactose, fructose, maltose, succinate, pyruvate, malate, sucrose, lactate and glycerol) and nitrogen sources (peptone, yeast extract, beef extract, casein, malt extract) were purchased from Hi Media, Bombay (India). The $MgSO_4.7H_2O$, KH_2PO_4, NH_4Cl, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ and other metal salts were purchased from Qualigens, Bombay (India). All other reagents were used of analytical grade.

Media and culture conditions

The culture was maintained in medium containing 0.1% beef extract, 0.2% yeast extract, 0.5% peptone and 0.5% NaCl. Alkaline phosphatase fermentation was carried out in a modified medium (Nomoto *et al*., 1988; Prada *et al*., 1996) containing 1% glucose, 0.5% peptone, 0.1% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.002% KH_2PO_4, 0.5% NaCl. The pH of the medium was adjusted to 7.5 by dissolving the medium constituents in 0.1 M Tris-HCl buffer. Inoculum was developed by transferring one loop full of the organism from the slant culture to 50 ml production medium in 250 ml Erlenmeyer flask. The flask was incubated in an orbital shaker at 37±1˚C and 175 rev per min for 24 hours for inoculum development.

Statistical analysis

All the data were expressed as mean ± standard error from three independent experiments (n = 3) using Sigma Plot (version-8.0). Central composite experimental design and statistical analysis of the data were done by using Minitab statistical software package (version-14).

Production of alkaline phosphatase

Fermentations were carried out by adding 5% inoculum to 50 ml medium in 250 ml Erlenmeyer flasks and incubating in an orbital shaker at 37±1˚C and 175 rev per min for 72 hours. The fermentated medium was then centrifuged at 10,000g at 10˚C for 15 minutes and cell free supernatant was used for determination of alkaline phosphatase activity.

Enzyme assay

Alkaline phosphatase activity was measured spectrophotometrically by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate disodium salt (pNPP) at 415nm (Garen and Levinthal, 1960). The enzyme sample (0.1 ml) was added to 1.9 ml of p-nitro phenyl phosphate disodium salt solution (2 mg per ml in 1 M Tris-HCl buffer at pH 10.0) and the mixture was incubated at 50˚C for 20 minute. The reaction was terminated by adding 0.5 ml of NaOH solution (5 M) and the absorbance of the product p-nitrophenol were measured at a wavelength of 415nm using UV-Vis Spectrophotometer (Shimadzu) (Pandey and Banik, 2011). One unit of enzyme activity is defined as the amount of the enzyme catalyzing the liberation of 1 µmole of p-nitrophenol per minute.

Selection and optimization of nutritional constituents

A number of carbon and nitrogen sources were tested to study their effect on alkaline phosphatase production. Production medium (50 ml) were prepared by taking 1% (w/v) of different carbon sources viz. glucose, lactose, fructose, maltose, succinate, pyruvate, malate, sucrose, lactate, glycerol and 0.5% (w/v) of different nitrogen sources viz. peptone, yeast extract, casein, malt extract, NH_4Cl, $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ in each flask maintained at pH 7.5 and were inoculated with 5% of cell (24 hours old) suspension. The flasks were placed on a rotary shaker (175 rpm) for 72 hours at 37±1˚C. Alkaline phosphatase activity was determined in each flask after 72 hours of fermentation. In this experiment, different carbon sources were sterilized separately.

After selection and optimization of carbon and nitrogen sources by conventional method, a statistical experimental approach, central composite design (CCD) was applied to study the cumulative interactive effect of the macronutrients of the media and to optimize their concentration to enhance the production of alkaline phosphatase. After optimization of macronutrients using CCD, various metal salts such as $CaCl_2$, $CoCl_2$, $CuSO_4$, $MgSO_4$, $MnSO_4$, NaCl and $Pb(NO_3)_2$ with varying concentration from 0.02 – 1.0% (w/v) were applied with optimized macronutrients and alkaline phosphatase production was carried out at 37±1˚C and 175 rev per min for 72 hours. The pH of the medium was adjusted to 7.5 by dissolving the medium constituents in 0.1 M Tris-HCl buffer.

Results and discussion

Effect of different carbon sources and nitrogen sources on production of alkaline phosphatase

Different carbon sources (1% w/v) were used in fermentation medium and it was found that glucose is the most suitable carbon source for *B. licheniformis* alkaline phosphatase production (Figure 1). As a carbon source, glucose has been used for various other microorganisms (Hydrean *et al*., 1977; Kumar *et al*., 1983; Sharipova *et al*., 1998) for production of alkaline phosphatase. Different nitrogen sources (0.5% w/v) were used in fermentation medium and it was found that the combination of peptone and yeast extract (5:1) show better effects for the production of alkaline phosphatase (Figure 1).

Although complex nitrogen sources are usually most preferable because these are metabolized to produce amino acids, nucleic acids, proteins and cell wall components which may provide all essential to a microbial systems for the production of enzymes. Peptone and yeast extract have been reported as suitable nitrogen source for alkaline phosphatase production (Yamane and

Maruo, 1978; Nomoto *et al*., 1988; Prada *et al*., 1996; Nesmeyanova *et al*., 1997; Dhaked *et al*., 2005).

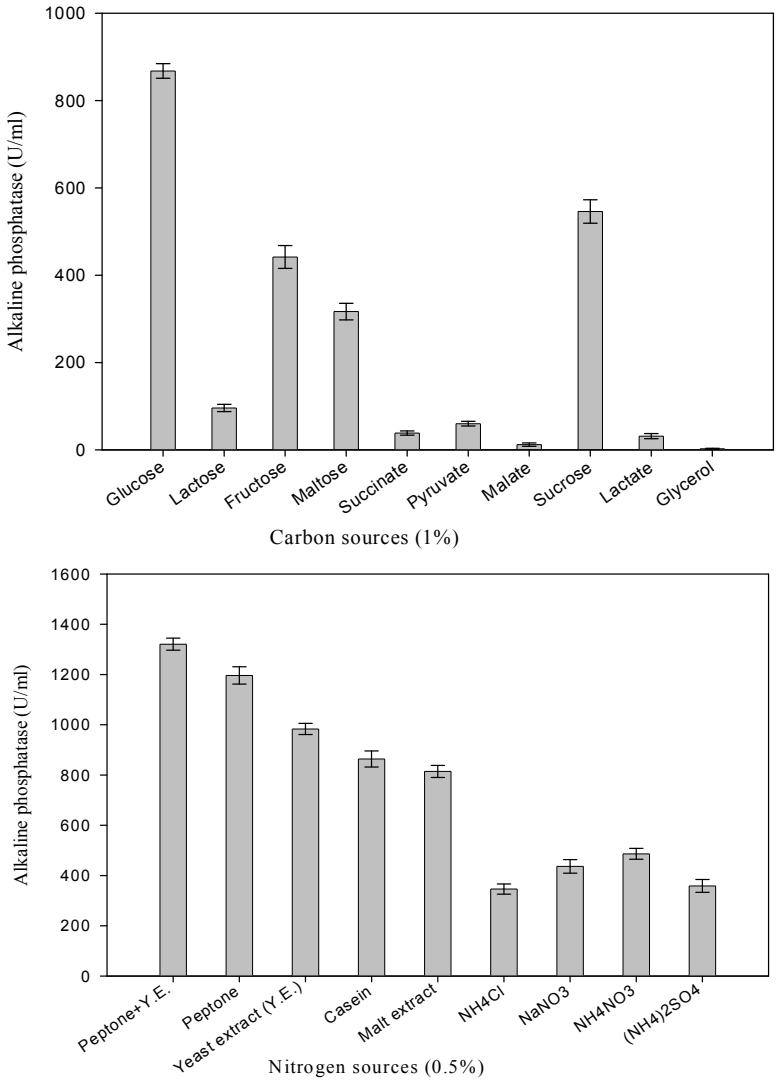

Fig. 1. Selection of carbon and nitrogen sources on production of alkaline phosphatase (U/ml)

Comparison of cell growth and alkaline phosphatase production at various initial glucose concentrations

Glucose consumption with comparison to cell growth and alkaline phosphatase production was carried out simultaneously to study the substrate inhibition and optimizing the substrate level. Glucose consumption was measured by DNS method (Miller, 1959) at different fermentation time interval by applying different concentrations of glucose from 1–5% (w/v), keeping the other parameters at constant. The remaining glucose concentrations and alkaline phosphatase activity were measured simultaneously at 24 h of fermentation time interval. Dry cell mass (gram per liter) of *B. licheniformis* were measured at 12 h of fermentation time. The results (Figure 2 and 3) indicate that 2% of glucose was completely utilized till 60 h of fermentation.

However, 3–5% of glucose was not completely consumed and remained present in the fermentation media. It was clearly proved that 2% of glucose is optimum concentration for maximum cell growth and alkaline phosphatase production. It is evidently proved from the results that *B. licheniformis* cell growth catalytically repressed by high glucose concentration (>2%). High concentration of substrates or products often lead to inhibitory effect which results in poor utilization of the substrates which in turn decrease both product yields and fermentation rates. Therefore, 2% of glucose was obtained as optimum concentration for better cell growth and alkaline phosphatase production. The concentration of glucose, peptone and yeast extract was further optimized by using central composite design (CCD) for getting precise level of cumulative effect to obtain high yield of alkaline phosphatase.

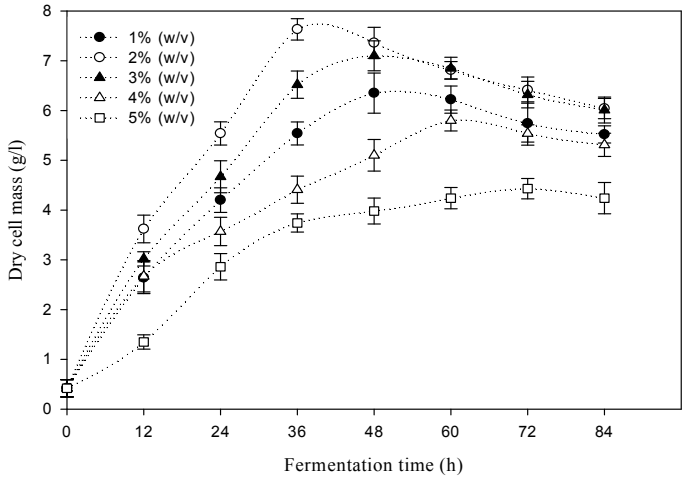

Fig. 2. Comparison of cell growth pattern at various initial glucose concentrations

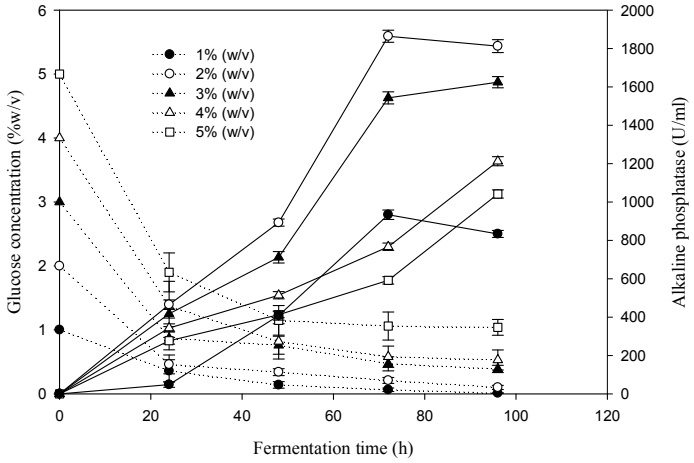

Fig. 3. Glucose consumption pattern with comparison to alkaline phosphatase production (U/ml)

Optimization of concentration of macronutrients using CCD

Among various carbon and nitrogen sources used, glucose, peptone and yeast extract were found to be the most suitable macronutrients for alkaline phosphatase production. The concentration of glucose, peptone and yeast extract was optimized using the central composite design (CCD). Optimization of the media constituents for overproduction of alkaline phosphatase by *B. licheniformis* was done by CCD, where a 2^3 factorial design was employed with 20 experiments. These 20 experiments performed with different combinations of three independent variables. Concentration levels of the three macronutrients glucose, peptone and yeast extract were considered as the three independent variables for optimization. The concentration of other media constituents was kept constant throughout the investigation. CCD was applied to determine the optimum concentration of three macronutrients. The effect of three macronutrients glucose, peptone and yeast extract on the production of alkaline phosphatase was studied at five experimental levels: -α, -1, 0, +1, +α, where α = $2^{n/4}$, here n was the number of variables and 0 corresponded to the central point. The levels of factors used for experimental design was glucose 1-3%, peptone 0.5-1.5% and yeast extract 0.1-0.2% low and high level respectively. The actual level of each factor was calculated by the following equation (Paul *et al*., 1992).

$$\text{Coded value} = \frac{\text{Actual level} - (\text{high level} + \text{low level})/2}{(\text{High level} - \text{low level})/2} \qquad (1)$$

The experimental plan and levels of independent variables are shown in Table 1. Glucose had a lower limit of 0.318% and upper limits of 3.68%. Peptone was varied between 0.159 and 1.84%. The lower and upper limits of yeast extract were 0.066 and 0.234%, respectively. The response variable was fitted by a second order model in order to correlate the response variable to the independent variables. The general form of the second degree polynomial equation used in this study is:

$$Y= \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

Where Y is the predicted response, x_i, x_j are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. Analysis of variance (ANOVA), regression analysis was done and contour plots were drawn by using Minitab Statistical Software package (version-14). In order to search optimum combination of major components of the medium to enhance the alkaline phosphatase production, experiments were performed according to the CCD experimental plan (Table 1.). Twenty experiments were carried out from the design by applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the alkaline phosphatase production by *B. licheniformis*.

$$Y= 2337.26 + 299.05X_1 + 296.05X_2 + 139.88X_3 - 364.47X_1^2 - 126.00X_2^2 - 268.63X_3^2 + 127.19X_1X_2 - 208.01 X_1X_3 + 41.93X_2X_3 \quad (3)$$

Where Y is the predicted response variable, alkaline phosphatase activity (U/ml) and X_1, X_2 and X_3 the values of independent variables, glucose, peptoneand yeast extract respectively. Among the three macronutrients glucose has highest impact on alkaline phosphatase production as given by highest linear coefficient followed by peptone and yeast extract. The P values for linear and quadratic effect of glucose, peptone and yeast extract are most significant due to low P values (<0.05). The P value for interactive effect of glucose-yeast extract is most significant due to low P values (<0.05), for interactive effect of glucose-peptone and peptone-yeast extract were found to be less significant as the P values are above 0.05. Regression analysis of CCD criterion data for alkaline phosphatase is given in Table 2. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in alkaline phosphatase production with the designed nutrients level. Closure the value of R (multiple correlation coefficients) to 1, better the correlation between the observed and predicted values. In the present study the value of R

(0.932) revealed that the model could explain up to 93.2% variation of alkaline phosphatase production. The P value for lack of fit (0.404) indicated that the experimental data obtained fitted well with the model and explained the effect of glucose, peptone and yeast extract on alkaline phosphatase production by *B. licheniformis*. Analysis of variance for the alkaline phosphatase production obtained from this design is given in Table 3. Figure 4 show the 2D contour plots of alkaline phosphatase production for each pair of nutrient concentration by keeping the other nutrients constant. The 2D contour plots are the graphical representations 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 major constituents of media for alkaline phosphatase production as obtained from the contour plots are as follows: glucose - 2.39%, peptone - 1.35% and yeast extract - 0.15%.

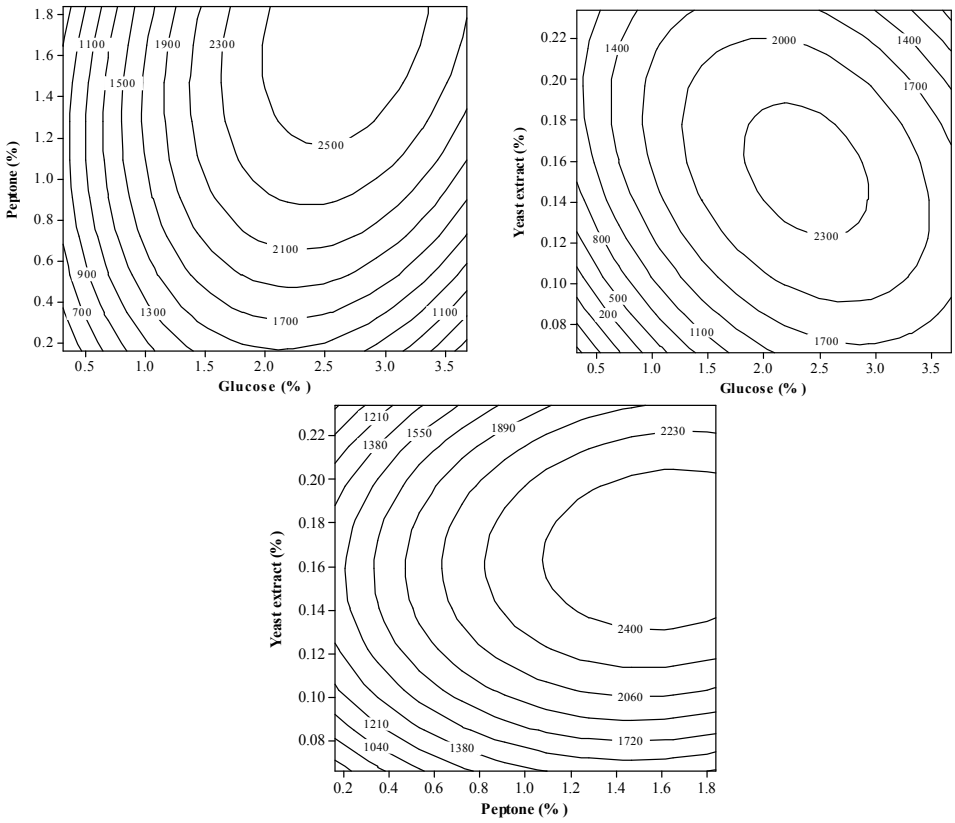

Fig. 4. Effect of glucose, peptone and yeast extract on alkaline phosphatase production (U/ml)

Table 1. Alkaline phosphatase production by *B. licheniformis* using macronutrients based on central composite design criterion

Glucose (%)	Peptone (%)	Yeast extract (%)	Activity (U/ml)	
			Experimental	Predicted
1.00000	1.50000	0.200000	1758.67	1837.78
2.00000	1.00000	0.150000	2480.68	2337.26
2.00000	0.15910	0.150000	1392.38	1483.00
3.00000	0.50000	0.100000	1723.21	1564.04
3.00000	1.50000	0.200000	2210.14	2274.26
2.00000	1.00000	0.065910	1054.08	1342.22
2.00000	1.00000	0.150000	2497.58	2337.26
2.00000	1.00000	0.150000	2249.84	2337.26
2.00000	1.00000	0.150000	2014.13	2337.26
2.00000	1.84090	0.150000	2456.18	2478.79
1.00000	0.50000	0.200000	1432.45	1416.20
3.00000	1.50000	0.100000	2390.47	2326.66
2.00000	1.00000	0.150000	2513.08	2337.26
1.00000	1.50000	0.100000	1240.50	1058.15
1.00000	0.50000	0.100000	948.48	804.30
3.68179	1.00000	0.150000	1814.27	1809.32
0.31821	1.00000	0.150000	685.25	803.43
3.00000	0.50000	0.200000	1241.63	1343.91
2.00000	1.00000	0.234090	1987.63	1812.71
2.00000	1.00000	0.150000	2287.68	2337.26

Table 2. Regression analysis of central composite design criterion data for alkaline phosphatase production by *B. licheniformis*

Term	Coef	SE Coef	T	P
Constant	2337.26	84.83	27.554	0.000
Glucose (%)	299.05	56.28	5.314	0.000
Peptone (%)	296.05	56.28	5.260	0.000
Yeast extract (%)	139.88	56.28	2.485	0.032
Glucose (%)*Glucose (%)	-364.47	54.79	-6.653	0.000
Peptone (%)*Peptone (%)	-126.00	54.79	-2.300	0.044
Yeast extract (%)*Yeast extract (%)	-268.63	54.79	-4.903	0.001
Glucose (%)*Peptone (%)	127.19	73.53	1.730	0.114
Glucose (%)*Yeast extract (%)	-208.01	73.53	-2.829	0.018
Peptone (%)*Yeast extract (%)	41.93	73.53	0.570	0.581

R^2 = 93.2%

Table 3. Analysis of variance for alkaline phosphatase production by *B.licheniformis* using central composite design criterion

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	5945403	5945403	660600	15.27	0.000
Linear	3	2685530	2685530	895177	20.69	0.000
Square	3	2770253	2770253	923418	21.35	0.000
Interaction	3	489620	489620	163207	3.77	0.048
Residual Error	10	432577	432577	43258		
Lack-of-Fit	5	240942	240942	48188	1.26	0.404
Pure Error	5	191635	191635	38327		
Total	19	6377979				

DF, Degrees of freedom; Seq SS, sequential sums of squares;
Adj SS, adjusted sums of squares; Adj MS, adjusted mean square.

Effect of various metal salts on alkaline phosphatase production

The results of experiments (Figure 5) clearly demonstrate that the alkaline phosphatase production increased by metal salts such as $CaCl_2$, $CoCl_2$, $MgSO_4$, $MnSO_4$,NaCl and decreased by $CuSO_4$ and $Pb(NO_3)_2$. $MgSO_4$, $CaCl_2$ and NaCl are most effective at concentration of 0.08%, 0.1% and 0.5% (w/v) respectively. However, $CoCl_2$ and $MnSO_4$ are very least effective around the concentration of 0.04%. By these results, it was observed that $MgSO_4$, $CaCl_2$ and NaCl are most significant metal salts for enhanced production of alkaline phosphatase. Therefore, $MgSO_4$; 0.08%, $CaCl_2$; 0.1% and NaCl; 0.5% (w/v) were selected as micronutrients with glucose; 2.39%, peptone; 1.35% and yeast extract; 0.15% (w/v) for high yield (2670.67 U/ml) of alkaline phosphatase. Available evidences suggest that metal ions such as Ca^{2+}, Co^{2+}, Mg^{2+}, and Mn^{2+} have profound effect on the production and stability of the alkaline phosphatase (Hulett and Jensen, 1988; Wojciechowski*et al*., 2002).

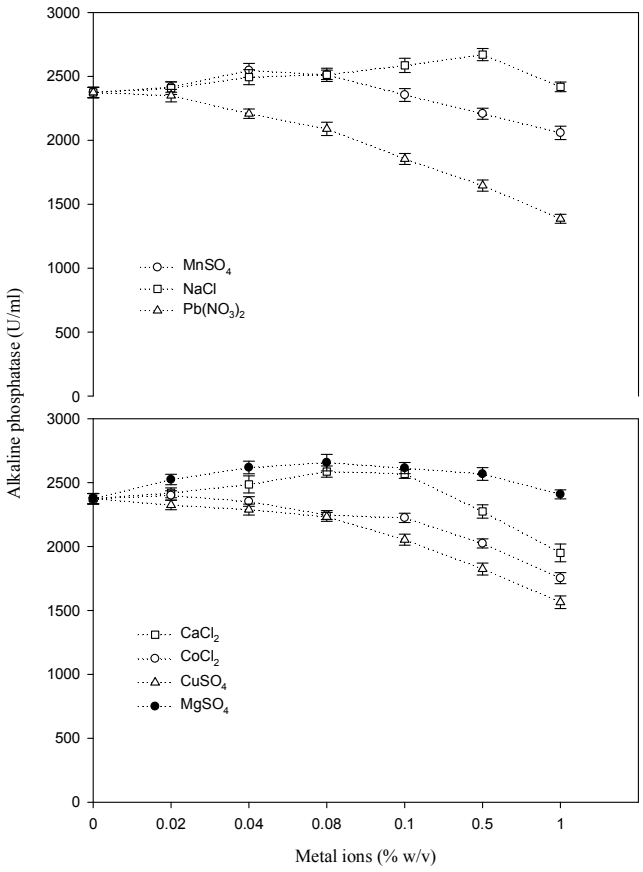

Fig. 5. Effect of various metal salts on alkaline phosphatase production (U/ml)

Conclusion

Present study deals the selection and optimization of nutritional constituents using conventional as well as statistically designed experimentation to obtain high yield of alkaline phosphatase. To the best of our knowledge there is limited information available in scientific literature concerning optimization of nutritional constituents for alkaline phosphatase production from *B. licheniformis*. The most significant nutritional constituents were selected conventionally and optimized their concentration using central composite design. The obtained results indicate that the most suitable nutrients composition for alkaline phosphatase production was glucose - 2.39%, peptone - 1.35%, yeast extract - 0.15%, $MgSO_4$ - 0.08%, $CaCl_2$ - 0.1% and NaCl - 0.5%. At these optimum levels of nutrients, alkaline phosphatase production of 2670.67 U/ml was obtained. The results of this study could be

used to design a suitable medium to get higher production of alkaline phosphatase.

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