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Optimization of bioprocess for enhanced production of alkaline protease by a *Bacillus subtilis* SHmIIIa through Plackett-Burman design

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Optimal conditions for the maximum production of alkaline protease by *Bacillus subtilis* SHmIIIa were evaluated by Plackett-Burman design. Nine process parameters namely, pH, temperature, agitation, inoculum, glucose, peptone, KH_2PO_4, $FeSO_4$ and tween 20 at two levels were selected for the design. Out of nine selected parameters, six parameters (pH 9.75, agitation 225 rpm, inoculum 3.5%, glucose 5.5 g/L, peptone 3.5 g/L and KH_2PO_4 1.5%, have shown significant influence on alkaline protease production. Optimization has resulted in spectacular enhancement in alkaline protease yield from 966 to 36000 EU/mg/ml with 37.26 fold increase.

Key words: *B. subtilis*SHmIIIa, alkaline protease, optimization, design of experiments, Plackett-Burman Design.

INTRODUCTION

Proteases (EC: 3.4. 21-24, 99) are one of the important groups of industrial enzymes with a share of more than 60% of the total global enzyme production (Beg et al., 2003). Alkaline proteases have several applications in a variety of industries such as detergents, foods, textile, pharmaceuticals, leather and in diagnostic reagents, recovery of silver from used x-ray films, wastewater treatment and clinical and medical applications (Kaur and Satyanarayana, 2005). International share market is expected to reach US $4.4 billion by 2015 (Oskouie et al., 2008). In view of the market potential, throughout the globe, the search for a potent alkaline protease producing bacteria which cater the needs of the current industrial sector is being taken up (Sen and Satyanarayana, 1993). Studies conducted so far suggest that microbial extracellular protease production is significantly influenced by media components including carbon, nitrogen sources and other environmental factors. A balance between the various medium components in production media is necessary for the enhanced protease production. The goal of any industrial process professional is to identify the factors that show a

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significant influence on the production. In any industrial setup, development of the cost effective fermentation process is necessary and also a challenging job. Optimization helps by minimizing the amount unutilized components. Statistical approaches have been made to identify the key factors to enhance the yield and reduce the cost of production to approximately 30-40% making the industrial process economically sustainable (Kaur and Satyanarayana, 2005). One-factor-at-a-time design approach has been quite popular with the researchers, mainly due to its simplicity of use. However the drawbacks are: it is time-consuming, requires more experimental runs and also cannot examine the interaction between factors. In this regard, the Plackett-Burman Design (PBD) is an efficient mathematical approach widely applied (Plackett and Burman et al., 1946). These experimental designs very clearly analyze the effects of variables on the response and pick up the essential factors). In light of the ever increasing demand for proteases, in the present study an attempt was made to maximize the alkaline protease production by *Bacillus subtilis* SHmIIIa mutantstrain using PBD in submerged and shake culture conditions.

MATERIALS AND METHODS

Microorganism

The bacterium used in the present studies was isolated from the slaughter house waste contaminated soils of Warangal (latitude 17° 58' N, longitude 79° 40' E and altitude 302 m (990 feet) located in Telangana state, India. The organism was isolated and tested for prospective alkaline protease production on alkaline skimmed milk agar (ASMA; Poly peptone 5 g/l; yeast extract 5 g/l; glucose 10 g/l; KH_2PO_4 1.0 g/l; $MgSO_4$ 0.02 g/l; skimmed milk 2 g/l; agar 18 g/l; Na_2CO_3 10 g/l) at pH 11.0, Protease production was readily observed by the formation of zone of clearance around the colony (Hegde et al., 2002).The culture of the mutant strain was maintained on Horikoshi basal medium (HBM) at pH 11 (Horikoshi, 1971).

Molecular identification

The efficient strain was tentatively identified as *Bacillus* sp based on morphological and biochemical characteristics and based on16S rRNA sequence homology and distance matrix, the organism was further identified as *Bacillus subtilis* sub sp. *Subtilis* strain DSM 10.

Mutagenesis with HNO_2

$NaNO_2$ with 0.1-0.3 M solution was prepared in carbonate buffer (0.02M pH 9.5). To one milliliter of the bacterial suspension 5 ml of the $NaNO_2$ was added. The solution was shaken thoroughly (Carlton and Brown, 1981). The one milliliter solution was withdrawn and diluted with 5 fold phosphate buffer (0.2 M, ph 7.1) to stop the reaction. A control was maintained by replacing $NaNO_2$ in acetate buffer with sterilized saline water. After fixed time interval, the tubes were spun at 6000 g for 15 min. The supernatant was discarded to remove nitrous acid from the pellet and ten milliliter of sterilized phosphate buffer was added to make the bacterial suspension. The tubes were respun for the three times to remove traces of nitrous acid. After washing the pellet was suspended in the same buffer. One milliliter of the bacterial suspension was plated on the ASMA medium and incubated at 30^{o}C for 48 h.

Enzyme production and assay

A single colony obtained on modified HBM with 1% skimmed milk was activated twice before the starter culture was prepared for seed inoculation. The starter culture was prepared by raising a cell suspension in a 500 ml baffled flask containing 100 ml of HBM medium (pH to 11.5±0.5) and incubated for 24 h on a shaker at 200 rpm. The starter absorbance was adjusted to 0.3 at 600 nm with modified Horikoshi medium. About 100 ml of ASMA medium with varying concentrations of the carbon, nitrogen and inducer sources was taken in flasks. Incubation was carried out for 24 h under the defined conditions. The cell-free supernatant recovered by centrifugation (10000 g, 10 min) was used as enzyme source. Extracellular alkaline protease activity was determined by the modified method of Kunitz (1947) and Yang et al. (1994).

About 0.1 ml of enzyme source was mixed with 0.9 ml of glycine-NaOH buffer (0.1M, pH 9) that contained 5 mg Hammerstein casein. Reaction mixture was incubated at 55□C for 10 min and later 2 ml of 5% trichloroacetic acid (TCA) was added to terminate the reaction. The contents were passed through No.2 filter paper (Whatman) to remove denatured proteins, filtrate was centrifuged at 10000 g for 10 min. The supernatant fraction was read at 275 nm. One unit of protease activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbency at 275 nm per min under the assay conditions. Specific activity was expressed as enzyme units per mg protein. Simultaneously, a blank without enzyme was used for comparison purpose.

Evaluation of cultural parameters for growth

The optimal growth conditions that is, pH, temperature, incubation time and inoculum levels for wild strain *B. subtilis* SH2 were evaluated by using MHBM broth.

Evaluation of nutritional parameters using OVAT method

The influence of various nutrients such as carbon and nitrogen sources was studied by one-variable-at-a-time method (OVAT method). Carbon sources (glucose, fructose, sucrose, maltose, lactose and starch) at the concentration of 1% (w/v) were evaluated while other components were maintained constant in the HBM composition. Similarly different nitrogen sources (tryptone, soy peptone, peptone, skim milk, yeast extract, beef extract) were analyzed at 0.5% levels while keeping other constituents at constant level.

Simulation of the Plackett-Burman Design

In order to identify the important variables affecting the enzyme production, PBD design with a set of two-level fractional factorial design with 12 experiments was employed. In this design, no main variables were mixed with any other variable, therefore, it efficiently achieves the goal and reduces the number of experiments required (Zhi and Feng et al., 2001; Zhng et al. 2013). PBD model was checked by F - test and goodness of fit by multiple regression analysis (De Coninck et al., 2000).The Design of experiments (DoE) methodology was utilized by employing the statistical analysis system (SAS) software version 9.0 (U.S.A).

Table 1. Optimized bioprocess parameters by OVAT method for *Bacillus subtilis* SHmIIIa.

Process variable	Mutant SHmIIIa	
	Formulated medium 1	
	Optimized level	EU/mg/ml
pH	9.56	3656
Incubation	66±6 h	
Temperature	43	
Inoculum	3%	
Rpm	200	
C	Glucose 5%	
N	Peptone 3%	
P&K	KH_2PO_4 1%	
Metal ion	$FeSO_4$0.2 mM	
Surfactants	Tween20 0.4%	

Validation of the experimental model

A random set of experiments were simulated to study the production of alkaline protease under the defined experimental conditions. The model was statistically validated with respect to all responses within the design space (Sasikumar and Viruthagiri, 2008). The conditions optimized from the model were further confirmed for accuracy by using the first order polynomial equation $Y= \beta_0 + \Sigma \beta_i X_i$ (i = 1, …. k) Where, *Y* is the estimated target function, *β0* is a constant, *β1 is* the regression coefficient, *X* is independent variable and *k* is the number of variables.

RESULTS

Microorganism

Based on the morphological, physiological and biochemical characteristics the bacterial isolate was tentatively identified as *Bacillus* sp. Further authentication was made by the 16S rDNA gene sequence analysis. The isolate SH2 showed 99% sequence homology with *B. subtilis* sub sp. *subtilis* strain DSM 10. Subsequently the isolate was mutated with HNO_2.

Evaluation of bioprocess parameters by OVAT methodology

In order to identify the essential process parameters and influence of various environmental, physiological and nutritional parameters on alkaline protease production OVAT methodology was adopted and the results are presented in Table 1. Our preliminary investigation revealed that FMI was found superior to HBM. Hence, the FM1 was considered as ideal for improved protease yield under the defined experimental conditions for the mutant strain.

Plackett-Burman design

Optimization of the fermentation conditions to maximize enzyme production was performed by the Plackett-Burman Design. A total of nine parameters were selected based on the OVAT method. The PBD simulation consisted 12 experiments at a resolution of 3. Each independent variable was investigated at a high (+1) and a low (-1) level which represent two different nutrient concentrations as shown in Table 2. The yield of the alkaline protease production by the present strain in defined fermentation conditions was studied and the results are précised in the Figure 1. Theyield of the enzyme was determined based on the t- values of the significant ingredients (Devendra and Pravin, 2010).

Determination of significance of each variable

The *p*-value serves as a tool for identification of significance of each variable and measures the probability magnitude coefficient of the process variability. The components were screened at the confidence level of 95% on the basis of their effects. Table 3 represents the results with respect to *t*-value, *p*-value and confidence level of each component. The present PBD model suggests that the componentsX1 pH; X3 agitation; X4 inoculum; X5 glucose; X6 peptone and X7 $KH_2PO_4$are significant or effective in alkaline protease production (Figure 1).

Validation of the model terms

The model was assessed using multiple regression equation (Myers and Montgomery, 2002) (Table 4). The model has shown 99.90 of the correlation between the model terms and the alkaline protease production. Hence, the model terms and alkaline protease production were strongly correlated (Palvannan and SathishKumar, 2010).

DISCUSSION

In general, the requirements of different bacterial strains for fermentation process differ. This usually depends upon the adaptive nature of the organism (Moorthy and Baskar et al, 2013; Pathak and Deshmukh, 2012). The regulatory mechanism behind the enhanced enzyme production is not yet understood. However, the contribution of the every physical and nutritional factor for optimal response should be known by performing OVAT method (Jeevan Chandra, 2013).Therefore, the role of individual factor has been taken into consideration for building a model process for optimized response (Moon and Parulekar, 1991; Periasamy et al., 2013). Through statistical analysis of the above model it is evident that out of nine parameters tested only six viz., pH; agitation; inoculum; glucose; peptone and KH_2PO_4 have shown a significant influence on enzyme production. The

Table 2. Placket Burman Design details of variables for production of alkaline protease by *Bacillus subtilis* SHmIIIa

Run	pH	Temp	Agitation	Inoculam	Glucose	Peptone	KH_2PO_4	FeSO4	Twntwnty	Protease
1	10	43	250	3	5	3	2	0.4	0.4	25734
2	10	45	200	4	5	3	1	0.4	0.4	1673
3	9.5	45	250	3	6	3	1	0.2	0.4	1243
4	10	43	250	4	5	4	1	0.2	0.2	26371
5	10	45	200	4	6	3	2	0.2	0.2	1435
6	10	45	250	3	6	4	1	0.4	0.2	21826
7	9.5	45	250	4	5	4	2	0.2	0.4	16363
8	9.5	43	250	4	6	3	2	0.4	0.2	1503
9	9.5	43	200	4	6	4	1	0.4	0.4	1232
10	10	43	200	3	6	4	2	0.2	0.4	21935
11	9.5	45	200	3	5	4	2	0.4	0.2	20435
12	9.5	43	200	3	5	3	1	0.2	0.2	1373

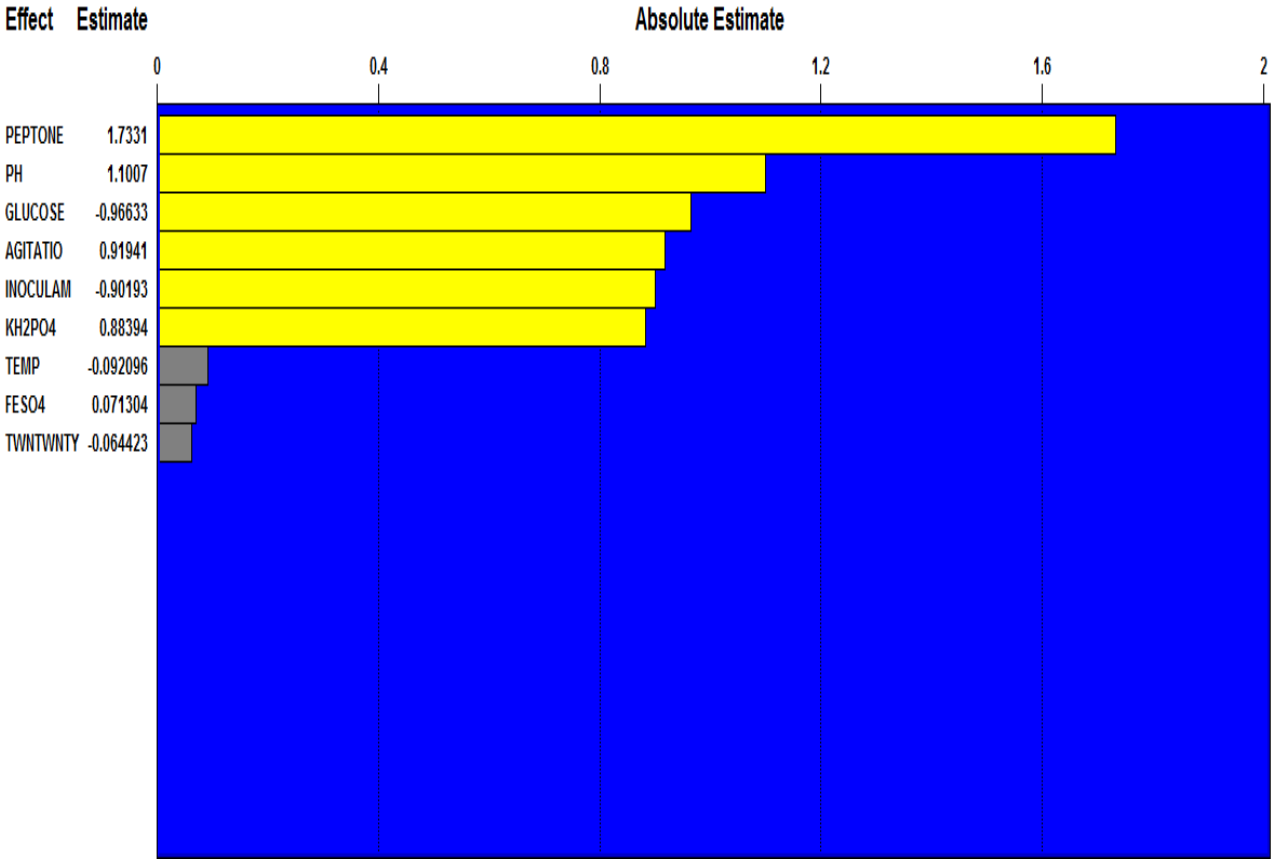

Figure 1. Pareto chart showing the important effects on alkaline protease production by Plackett-Burman Design for*Bacillus subtilis* SHmIIIa.

probability *P* value for lack of fit (0.022395) indicated that the experimental data obtained with the model is very good. In addition, the parameters have shown a direct influence on enhanced production by native strain in shake cultures (Cazetta et al., 2007).

Physiological adaptation of the strain shows a direct influence on the cellular activity of enzyme secretion. pH has strong influence in many enzymatic processes and transport of several molecules across the cell membrane. Difference in media pH alters acid-base equilibria and

Table 3. ANOVA of the Plackett-Burman Design for alkaline protease production by *Bacillus subtilis* SHmIIIa.

	Master model					Predictive model				
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
PH	1	3.634563	3.634563	324.5909	0.003067	1	3.634563	3.634563	240.5611	0.0001
Temp	1	0.025445	0.025445	2.272418	0.270698					
Agitation	1	2.535926	2.535926	226.4753	0.004386	1	2.535926	2.535926	167.8456	0.0001
Inoculum	1	2.440452	2.440452	217.9488	0.004557	1	2.440452	2.440452	161.5264	0.0001
Glucose	1	2.801387	2.801387	250.1827	0.003973	1	2.801387	2.801387	185.4156	0.0001
Peptone	1	9.011211	9.011211	804.7618	0.00124	1	9.011211	9.011211	596.4257	0.0001
KH2PO4	1	2.344055	2.344055	209.3399	0.004743	1	2.344055	2.344055	155.1462	0.0001
FESO4	1	0.015253	0.015253	1.362167	0.36349					
Twntwnty	1	0.012451	0.012451	1.111953	0.40224					
Model	9	22.82074	2.535638	226.4496	0.004404	6	22.7676	3.794599	251.1534	0.0001
Error	2	0.022395	0.011197			5	0.075543	0.015109		
Total	11	22.84314				11	22.84314			

Table 4. Validation of the PBD model for alkaline protease production by *Bacillus subtilis* SHmIIIa

Analysis of coeffient of variance	Master model	Predictive model
RMSE	0.105818	0.122917
R-square	99.90%	99.67%
Adjusted R-square	99.46%	99.27%
Coefficient of Variation	1.227784	1.42619

fluxes of various nutrients in cell interior. The common optimum pH range for protease production among alkaliphilic and halo alkaliphilic organisms lies between 9 to 10 (Patel et al., 2006). The present investigations suggest that the test organism grows profusely at pH 9.75, which markedly specifies the alkaliphilic nature of the strain. Present observations are in agreement with the earlier studies carried out by Bhaskar et al. (2007).Krulwich (1995) has studied the facultative alkaliphile *Bacillus firmus*OF4 and pointedthat Na1/H1 anti-porter enables cells to adapt to shift in pH and to maintain the external pH in the most alkaline range of pH for growth.

The agitation rate has indirect relation with the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by means of variations in the aeration rate and agitation speed (Chu et al., 1992). The variation in the agitation speed influences the extent of mixing in the shake flasks and distribution of the substrates evenly in the medium makes them available for the growth and protease production (Moon and Parulekar, 1991*).*Hence, considerable improvement in the production of the alkaline protease can be achieved with the proper maintenance of the agitation. In the present investigation, 225 rpm of the speed agitation was found to be ideal for the enhanced protease production. These results concur with the earlier studies made by Sudhir and Ashis (2010).

A high inoculum size improves biomass production, while low inoculum size is generally unsupportive for the growth as well as enzyme secretion. A moderate inoculum size was suggested to the increased protease production, which may be due to the higher surface area to volume ratio resulting in increased protease production (Michalik et al., 1995).In the present investigations, the alkaline protease production steadily increased with increase in inoculum size and reached maximum at 3.5% level of inoculum production. Similar observations were made by Reddy et al. (2008).

Nutritional factors are the key contributors with a great extent of capacity for enhanced production of alkaline protease. The alkaline protease comprises 15.6% nitrogen and thus its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole et al., 1988). In the preset study, peptone and glucose have shown a significant influence on protease production and are considered as the prime factors of the entire process in the master model. For alkaline protease production, the complex nitrogen sources are usually preferred over the simple inorganic. This is just because of the feedback inhibition imposed by simple nitrogen sources. The same situation was

Table 5. Factors optimized through PBD for enhanced alkaline protease production by *Bacillus subtilis* SHmIIIa.

Factor	Setting	EU/mg/ml
pH	9.75	
Temperature	44°C	
Agitation	225 rpm	
Inoculum	3.5%	
Glucose	5.5 g/l	36000
Peptone	3.5 g/l	
KH_2PO_4	1.5 g/l	
$FeSO_4$	0.3 mM	
Tween20	0.3%	

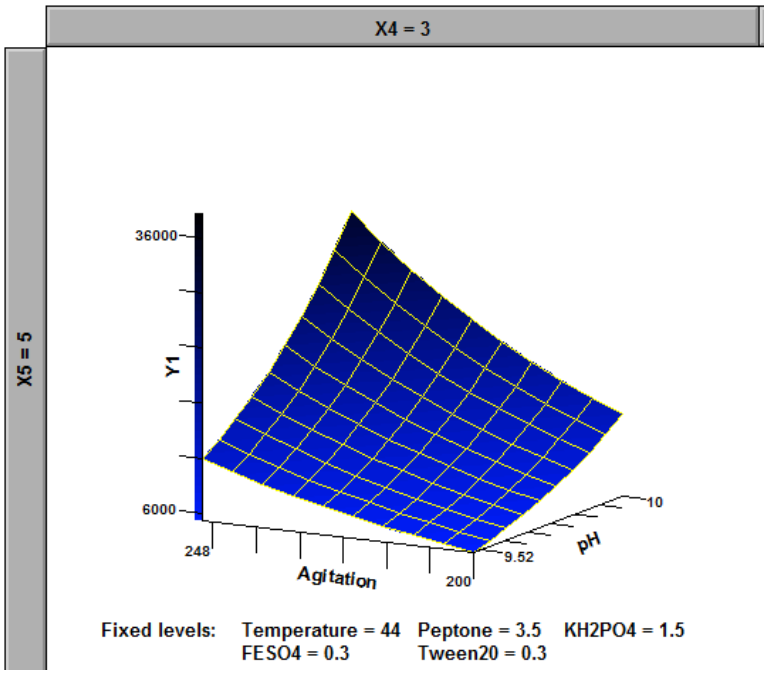

Figure 2. Response surface plot for production of alkaline protease by *Bacillus subtilis* SHmIIIa.

encountered with carbohydrates. Low levels of alkaline protease production were reported in the production medium (Chandrasekaran and Dhar, 1983). Present observations are similar to those of the studies made by Chaphalkar and Dey (1994) and Sen and Satyanarayana (1993).

Validation of the predicted model for enhanced alkaline protease production

The t-values of the process variables are 18.01641, 15.0491, 28.36832, 14.46858 and 1.167119 for pH, agitation, peptone, K_2HPO_4 and $FeSO_4$ respectively, showing a significant improvement. These findings indicate that these variables are very important for optimization of alkaline protease production. The R^2 and adjusted R^2 are 99.90 and 99.46% respectively which indicate a high correlation between the observed values of master model and the values of predicted model. This specifies that regression model provides an excellent correlation between the independent variables (factors) and the response (alkaline protease production) (Table 5).

The wild strain (SH2) at primary screening level has shown only 966 EU of alkaline protease production, however on mutagenesis it has increased to 3688 EU of enzyme production with 3.8 fold increase. The same bacterial strain in the final phase of optimization through PBD has shown a dramatic enhancement to 36000 EU with 37.26 fold increase (Figure 2).

Conclusions

The present investigations were primarily aimed at identifying the important factors which contribute to the enhanced protease production. The PBD has allowed the evaluation of main variables of the enzyme bioprocess. The PBD model adopted in the present investigations explained the effect of pH, agitation, inoculum, glucose, peptone and KH_2PO_4 on alkaline protease production by *B. subtilis* SHmIIIa. Further, investigations on characterization and stability of the alkaline protease under harsh industrial conditions are under progress.

Conflict of interests

The authors did not declare any conflict of interest.

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