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PLACKETT-BURMAN DESIGN FOR SCREENING MEDIA COMPONENTS FOR ALKALINE PROTEASE PRODUCTION FROM *BACILLUS LICHENIFORMIS* THROUGH SUBMERGED FERMENTATION

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ABSTRACT

Bacillus licheniformis SCJ4, a marine bacteria isolated from highly polluted area of Lucknow, showed a high potency for contemporaneous production of alkaline protease enzyme. In the current study, Plackett-Burman factorial design was applied to evaluate culture conditions affecting the production of enzyme. Analysis of Plackett-Burman design results revealed that, the most significant variables affecting alkaline protease production were glucose, K_2HPO_4, $MgSO_4$ and $CaCl_2$ with more than 6 folds increase in enzyme activity than basal medium (1185 Uml-1min-1 after 48h).

Keywords: *Bacillus licheniformis*, fractional factorial design, enzymes

INTRODUCTION

The increase of commercial demands for protease enzyme ranked them among the most important industrial enzymes in use today **[1, 2]**, they account for over 60 and 25% of the total industrial enzyme market; correspondingly **[2-4]**. The two enzymes are involved in many industrial applications such as pharmaceutical agents, food products, and laundry detergents **[2,5-7]**. Despite their wide spread in almost all living organisms, including plants, animals, and microorganisms, the special characteristics of microbial enzymes generally meet industrial demands **[8-10]**. The high cost of enzyme production is one of the major bottlenecks facing industrial enzyme producers, as 30-40% of the production cost of many industrial enzymes is attributable to the cost of the cultivation medium. More effort to be done toward optimization of enzymes production medium is expected to reduce production

costs drastically, also using the potent organism with high enzymes production potential is essential strategy to reduce the production cost. One of the recently emerged sources of industrially important enzymes is marine microorganisms. Highly contaminant marine niches with different xenobiotics and heavy metals may represent a good source for industrially important microbial enzymes with special characteristics.

Fermentation studies are often carried out using classical methods of experimentation; with one- factor-at-a-time changed while all others are held constant. This strategy takes more time and effort and consequently does not guarantee getting the optimal conditions since it neglects interaction between variables. Statistical-mathematical designs such as Plackett–Burman design present a more balanced alternative to the one-factor-at-a-time approach to fermentation improvement. For example, instead of testing carbon and nitrogen sources in two separate experiments; one may test all combinations of carbon and nitrogen simultaneously. Plackett-Burman designs comprise one type of two level screening designs. These designs allow for the study of up to (n-1) factors with (n) trials. Low and high factor settings are coded as –1 and +1, respectively. Application of the statistical experimental designs has become a very effective tool for optimization of enzymes production process and reported in many articles.

Bacillus licheniformis species is well known for its ability to assimilate methanol as a sole carbon source, hence its name however, to the best of our knowledge no reports are available for enzymes' production from this strain. The present study reports the production of alkaline protease by the marine *Bacillus licheniformis* SCJ4 locally isolated from Lucknow.

MATERIALS AND METHODS

Microorganism isolation, identification and cultivation conditions

Bacterial strain used in the current study, *Bacillus licheniformis* SCJ4, was isolated and identified through our previous work conducted on Gomti River water samples collected from the Lucknow. The isolate was routinely cultivated on Luria-Bertani liquid medium (LB) with the following composition (g/l): Tryptone, 10; yeast extract, 5 and sodium chloride, 5. pH was adjusted to 7.5 before autoclaving. LB liquid medium was used as a preculture medium in this study.

Enzyme production conditions

Alkaline protease production were carried out in 250ml Erlenmeyer flasks containing 50ml liquid artificial seawater medium contained (g/l): NaCl, 27; MgSO4.7H_2O,6.6; $MgCl_2$.6H_2O, 5.6; $CaCl_2$.2H_2O, 1.5; KNO_3, 1.0; KH_2PO_4, 0.07; $NaHCO_3$, 0.04; 20.0ml of Tris-HCl buffer (1.0M, pH 7.6), 1ml of chelated iron solution (g %: FeCl3.4H2O, 0.24; EDTA, 14.6) and 1ml of trace metal solution (mg %: H3BO3, 60.0; MnCl2.H2O,

40.0; (NH4)6Mo7O24.4H2O, 37.0; CuCl2.2H2O, 4.0; ZnCl2, 4.0; CoCl2.6H2O, 1.5). In screening for alkaline protease activity, the artificial sea water medium supplemented with (gelatin) were inoculated separately with 1% of 12h old broth culture then incubated at 30°C under shaking conditions (200 rpm). After specified cultivation time the culture broth was centrifuged at 10,000 rpm for 10 minutes and the cell free supernatant was used as source of crude enzyme.

Quantitative determination of alkaline protease activity

Estimation of alkaline protease activity was carried out according to **[7]** where, one ml from 1% of bovine milk casein in 50 mM glycine-NaOH buffer pH 10 was incubated with 1ml diluted enzyme at 50ºC. Then after incubation, the reaction was stopped by addition of 3ml from 5% TCA solution. The remaining undigested casein was precipitated by centrifugation at 13,000 rpm for 10 min. then 1ml supernatant was measured spectrophotometrically at 280nm. A standard curve was prepared using L-Tyrosine. One unit of protease activity was defined as the amount of enzyme that yields the equivalent of 1 µmol of L-Tyrosine per minute under the assay conditions.

Basal medium for enzymes production

For selecting a basal medium for enzymes production, different pre-optimized alkaline protease production media were initially tested for their ability to support both alkaline protease production, the media with the following composition (g/l) were used: Medium coded M1: Casamino acid, 10; chicken feathers, 10; K2HPO4, 3; KH2PO4, 2; Na2SO4, 2; and MgSO4, 0.1**[9]**. Medium coded M2: Chicken feathers, 2; casin, 2; yeast extract, 10; peptone, 20; KH2PO4, 1; and MgSO4, 0.1[30]. Medium coded M3: Chicken feathers, 5; soybean meal, 10; K2HPO4, 3; MgSO4.7H2O, 0.5; NaCl, 0.5; and CaCl2.2H2O, 0.5 **[1]**. Medium coded M4: Citric acid, 10; NaNO3, 10; K2HPO4, 5, MgSO4.7H2O, 0.3; CaCl2.2H2O, 0.2; NaCl, 5.0 **[8]**. Medium coded M5: Glucose, 10; peptone, 10; yeast extract, 20; KH2PO4, 0.05; MnCl2.4H2O, 0.015; MgSO4.7H2O, 0.25; CaCl2.2H2O, 0.05; and FeSO .7H O, 0.0132. The media pHs were initially adjusted to 7.0 before autoclaving. The enzymes activities were measured after 48h cultivation period and temperature (30ºC).

Plackett-Burman design

Plackett-Burman experimental design was used to evaluate the relative significance of fifteen culture factors affecting both alkaline protease and α-amylase production by *Bacillus licheniformis* EGY-SCJ4. The tested variables were tested at two levels high (+) and low (-) concentrations in addition to the middle concentration (0) **(Table 2)**. The matrix design of fifteen screened factors in sixteen combinations was shown in **Table 3**. All trials were done in 250ml Erlenmeyer

flasks containing 50ml of the medium. Plackett-Burman screening design depends on the first order model:

$Y = \square_0 + \square\square_i x_i$ (1)

In this model Y representing the response (alkaline protease), $\square_0$ is the model intercept, $\square_i$ is the variable estimate and x_i represents the variable. The Pareto plot best demonstrate the results of Plackett-Burman design that illustrates the absolute relative significance of variables independent on their nature.

Plackett-Burman design data analysis

Multiple linear regressions, function in Microsoft Excel 2007, were used in analyzing the data of enzymes activities to estimate t-value, P-value and confidence level. The significance level (P-value) is determined using the Students t-test. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. If this probability is sufficiently small, the idea that the effect was caused by varying the level of the variable under test is accepted. Confidence level is an expression of the P-value in percent.

Table 1: Plackett-Burman experiment 15 variables and its tested levels

Variables	Variable code	Low level (-1)	Middle level (0)	High level (+1)
Chicken feathers (%)	X1	0.20	0.50	1.00
Chicken feathers (%)	X2	0.20	0.50	1.00
Soybean flour (%)	X3	0.20	0.75	1.00
Peptone (%)	X4	0.10	0.25	0.50
Yeast extract (%)	X5	0.10	0.25	0.50
K_2HPO_4 (%)	X6	0.10	0.20	0.30
$MgSO_4.7H_2O$ (%)	X7	0.01	0.025	0.05
NaCl (%)	X8	0.30	0.50	0.70
$CaCl_2$ (%)	X9	0.00	0.025	0.05
$FeSO_4$ (%)	X10	0.00	0.0005	0.001
Culture pH	X11	6.00	7.0	8.00
Temperature (°C)	X12	30.0	35.0	40.0
Incubation time (h)	X13	24.0	36.0	48.0
Inoculum size (%)	X14	1.00	2.00	3.00
Inoculum age (h)	X15	12.0	18.0	24.0

Table 2: Randomized Plackett-Burman experimental design for evaluating factors influencing alkaline protease production

Trial	Chicken feathers	Glucose	Soybean flour	Peptone	Yeast extract	K2HPO4	MgSO4	NaCl	CaCl2	FeSO4	pH	Temperature	Incubation time	Inoculum size	Inoculum age	Protease activity Uml-1min-1
1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	420.6
2	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	490.8
3	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	262.2
4	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	548.4
5	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	253.8
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	532.8
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	187.2
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	694.2
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	585.6
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	198.6
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	103.2
12	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	160.2
13	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	555.6
14	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	428.4
15	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	496.0
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	107.4
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	399.6
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	374.2
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	360.8

RESULTS AND DISCUSSION

Microorganism isolation, identification and characterization

In the course of a qualitative screening program for alkaline protease activity, sixty seven isolates from highly xenobiotic contaminant area of Lucknow **[2, 6]** were tested. From these isolates 24 (35.83%) showed alkaline protease activity. Based on quantitative determination of protease activity, the isolate coded SCJ4 which was isolated from river water sediments showed the highest potential for alkaline protease production in artificial river water (186.4 Uml-1min-1) and (31.5 Uml-1min-1) under assay conditions, respectively. The morphological and physiological characteristics of the selected isolate coded SCJ4 are presented in **Table 3** and showed that it belongs to the genus Bacillus and has the ability to utilize methanol and ethanol as sole carbon sources.

Different media used for testing protease production

Pandey *et al.* **[33]** have addressed the concept that there is no general medium for enzymes production by different microbial strains. Considering this fact, five different pre-optimized alkaline protease media were initially tested. Though, all tested media supported enzyme productions, except M4 which has well defined components suppressed both growth and enzyme productions **(Figure 1)**. These results are in agreement with many findings reported the enhancement of enzymes' production through complex carbon-nitrogen sources **[4-6]**. In contradiction, other studies reported a high protease yield in presence of ammonium sulfate, potassium nitrate or sodium nitrate as nitrogen source **[3, 7]**. The highest alkaline protease production (347.1 Uml-1min-1) after 48h was obtained in M3 medium. On the other hand, the highest amylase activity (48.4 Uml-1min-1after 48h) was measured in M5 medium.

In brief the production of enzymes by *Bacillus licheniformis* SCJ4 was achieved in all test media except the medium with well-defined components (M4). A high titer of protease enzyme was recorded in M3. Therefore it is necessary to optimize the production of each enzyme individually using M3 as basal medium for protease enzyme

Evaluation of the factors influencing alkaline protease production

For alkaline protease, the data in **Table 4** revealed a wide variation from 103.2 to 694.2 Uml-1min-1of protease activity. Analysis of data (main effects) of Plackett-Burman experiments implies a first order model. The main effects of the examined factors on the enzyme activity were calculated and presented graphically in **Figure 2**. Based upon the regression coefficients analysis of tested variables: chicken feathers, glucose, soybean flour, K_2HPO4, $MgSO_4$, NaCl, $CaCl_2$, culture pH, cultivation temperature

and cultivation time showed positive effect on alkaline protease activity, where peptone, yeast extract, FeSO4, inoculum size and inoculum age were contributed negatively. **Figure 3** shows the ranking of factor estimates in a Pareto chart. The results of a Plackett-Burman design analysis could be displayed in a very convenient way using Pareto chart.

Table 3: Morphological and biochemical characteristics of SCJ4 isolate

Test	Result
Cell shape	Rode
Gram stain	-ve
Motility	+ve
Growth on methanol (0.5%)	+ve
Growth on ethanol (0.5%)	+ve
Maximum growth temperature	50°C
NaCl growth range	0 - 7.5%
Gelatin liquefaction	+ve
Arginine	+ve
Urease	+ve
ONPG test	+ve
Citrate utilization	+ve
Indole production	-ve
VP test	-ve
Mannitol fermentation	-ve
Xylose fermentation	+ve

*ONPG=Hydrolysis of o-nitrophenyl-ß-d-galactopyranoside (ONPG

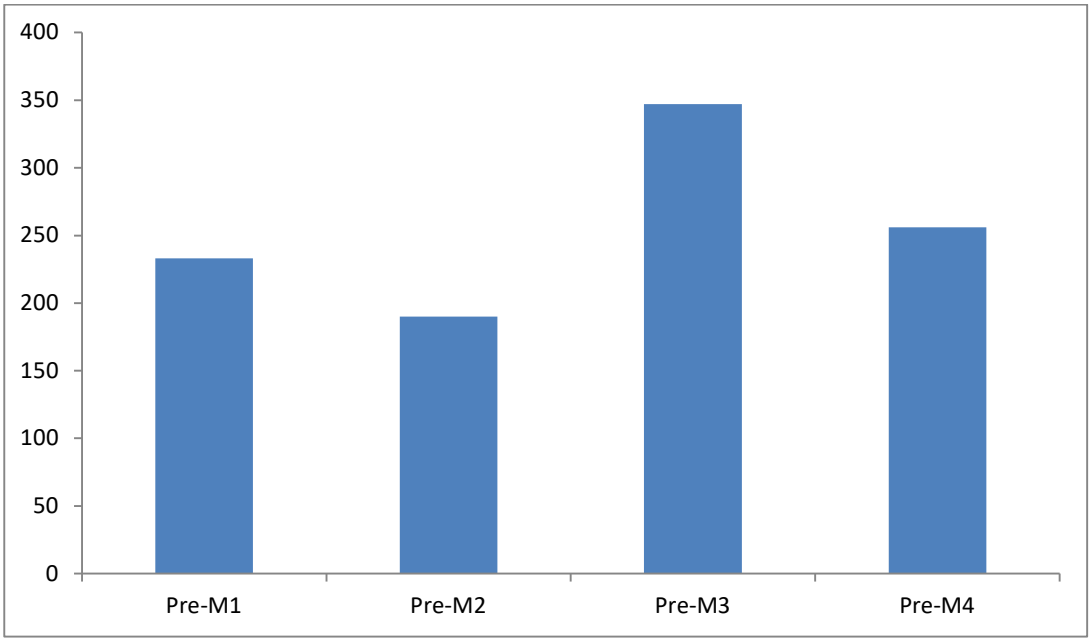

Figure 1: Effect of different pre-optimized medium on the alkaline protease production ($Uml^{-1}min^{-1}$)

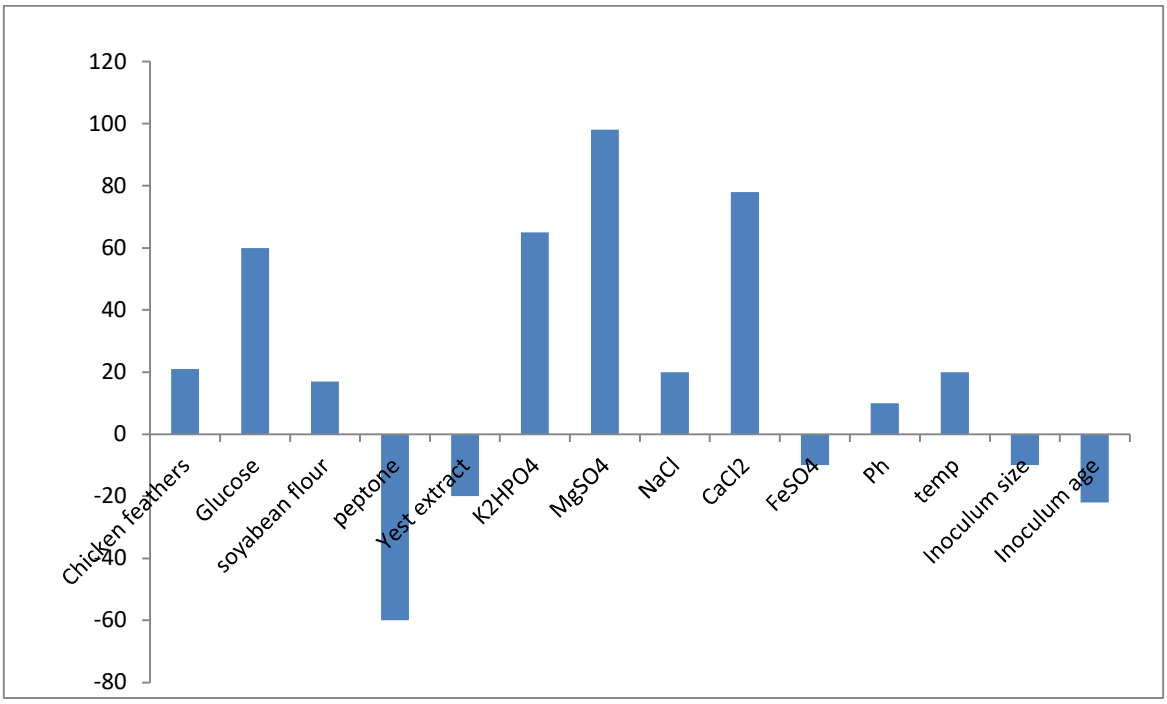

Figure 2. Effect of culture conditions on alkaline protease enzymes produced by *Bacillus licheniformis* SCJ4 based on Plackett-Burman design results

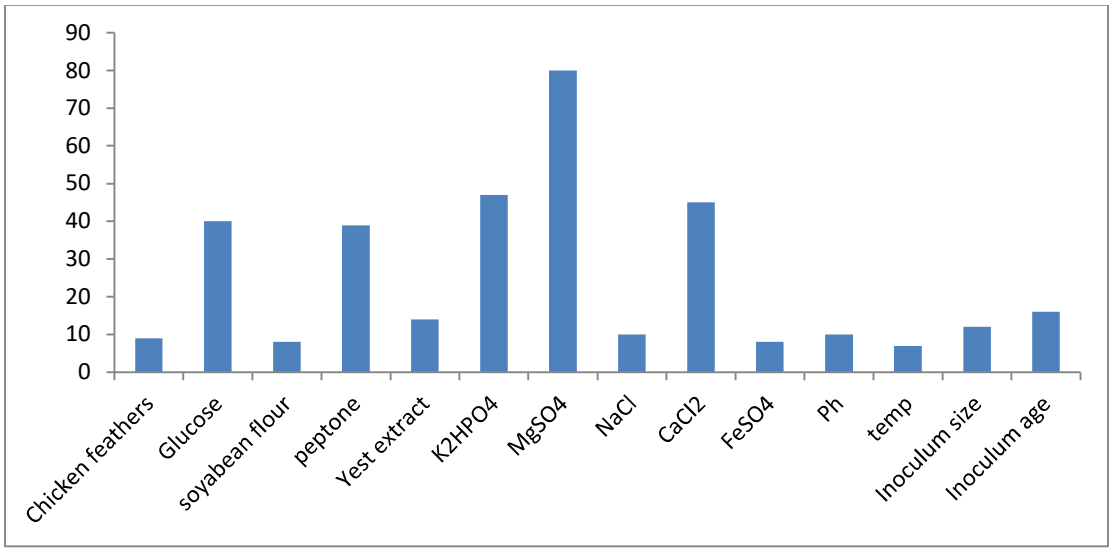

Figure 3: Pareto plot for Plackett-Burman parameter estimates of alkaline protease enzymes produced by *Bacillus licheniformis* SCJ4

Table 4: Alkaline protease activity

Variables	Protease enzyme			
	Coefficient	t-test	P-value	Confidence level (%)
Glucose	16.80	3.03	0.0564	94.36
Chicken feathers	65.62	11.83	0.0013	99.87
Soybean flour	4.30	0.77	0.4958	50.42
Peptone	-65.96	-11.89	0.0013	99.87
Yeast extract	-41.69	-7.52	0.0049	99.51
K2HPO4	80.04	14.43	0.0007	99.93
MgSO4.7H2O	114.75	20.69	0.0002	99.98
NaCl	24.97	4.50	0.0205	97.95
CaCl2	73.27	13.21	0.0009	99.91
FeSO4	-16.02	-2.89	0.0631	93.69
Culture pH	30.79	5.55	0.0115	89.85
Temperature	2.50	0.45	0.6826	31.74
Incubation time	41.22	7.43	0.0050	99.50
Inoculum size	-22.10	-3.98	0.0283	97.17
Inoculum age	-39.30	-7.09	0.0058	99.42

The full polynomial model describing the correlation between the 15 factors and the alkaline protease activity based on analysis reported in **Table 4**, could be presented as follows:

$Y_{activity} = 376.82 + 16.80X_1 + 65.62X_2 + 4.3\ X_3 - 65.96\ X_4 - 41.69X_5 + 80.4X_6 + 114.75X_7 - 24.97X_8 + 73.27\ X_9 - 16.02X_{10} + 30.79X_{11} + 2.5\ X_{12} + 41.22X_{13} - 22.1X_{14} - 39.30X_{15}$

From the confidence level of the variables, it was apparent that $MgSO_4$, K_2HPO_4, and $CaCl_2$ were the most significant variables positively enhancing the alkaline protease production. In contrast, FeSO4 suppressed the enzyme production. It was reported that Mg2+, Ca2+ and K+ salts induce protease production and increase its stability.

In this study, glucose, chicken feathers showed positive effect on alkaline protease production, this finding may be in accordance with others' studies that reported the enhancement of alkaline protease production from Bacillus sp. with glucose, and chicken feathers. Contrary to many findings stated, the high carbohydrates concentration, especially simple sugars, could suppress protease production, due to catabolic repression or severe decrease in cultivation pH as a result of acid productions upon utilization of simple sugars. The tested carbon–nitrogen sources, peptone and yeast extract, showed an adverse effect on enzyme production. Soybean flour was the only tested carbon–nitrogen sources showed a positive effect on protease production, this is in agreement with other studies reported high alkaline protease production using soybean flour as nitrogen source. Soybean flour is an agricultural waste with protein concentration ranged 51.2–53.2%; represent a good cost-effective fermentation medium ingredient, also using of soybean flour as nitrogen source decreases the nitrogen repression effect caused by using simple inorganic nitrogen

sources in culture medium45. On the other hand **[4, 9]**, reported that the usage of peptone and yeast extract as nitrogen sources maximize the enzyme production.

According to Plackett-Burman design results, a medium with the following composition (w/v%): Chicken feathers, 1; glucose, 1; soybean flour, 1; peptone, 0.1; yeast extract, 0.1; K_2HPO_4, 0.3; $MgSO_4$, 0.05; NaCl, 0.7; $CaCl_2$, 0.05 is expected to be near optimum for alkaline protease production by the tested isolate (SCJ4).

Based upon the adverse effect of peptone and yeast extract on alkaline protease production and for medium simplification, several media formulations have been tested to improve the formula of the previous medium. Peptone and yeast extract were omitted separately or in combination[2] from production medium. Other variables with positive main effect were used in its (+1) coded values, and variables with negative main effect values were used in its (–1) coded value according to the Plackett-Burman design results. The first formula, without peptone, showed alkaline protease activity of (970.5 Uml-1min-1). The second formula, without peptone, showed activity of (940.5 Uml-1min-1), where formula without peptone and yeast extract was the most potent one with activity of (1185 Uml-1min-1). According to these results, a medium of the following composition is expected to be optimum (g/l): glucose, 10; soybean flour, 10; K_2HPO_4, 3; $MgSO_4$, 0.5; NaCl, 7; CaCl, 0.5; inoculum size of 1% from 12 h age cells, where maximum enzyme activity was 1185 Uml-1min-1after 48h under shaking (200 rpm), at 30ºC and pH 8. This result represents 6.4 folds increase in protease activity, when compared to results obtained before applying the Plackett-Burman design.

According to these results, a medium of the following composition is expected to be near optimum (g/l): chicken feathers, 10; Glucose, 10; soybean flour, 10; peptone, 5; K_2HPO_4, 1; $MgSO_4$, 0.5; NaCl, 3; inoculated with 1% of 12h old culture, where maximum enzyme activity was 280.4 Uml-1min-1 after 48 h. under shaking (200rpm), at 40ºC and pH 6. This results presented about 8.9 folds increase in the enzyme activity, when compared to results in medium under basal conditions.

CONCLUSION

The present study investigated the factors affecting the production of alkaline protease *Bacillus licheniformis* EGY-SCJ4. This is considered the first reported study focused on optimization the production of protease enzymes by this isolate using experimental design. Finally, the enzyme yields were improved and reached to 6.4 -folds compared to the basal medium for alkaline protease.

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