ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Application of Plackett-Burman Design for Optimization of Alkaline Protease and α-amylase Production by the Marine Bacterium *Bacillus methylotrophicus* SCJ4

Hamada El-Gendi[1], Mohamed S. Azab[2], Nadia A. Soliman[1]* and Yasser R. Abdel-Fattah[1]*.

[1] Bioprocess Development Dept., Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technological Applications, Alexandria, Egypt.
[2] Botany and Microbiology Dept., Faculty of Science, Al-Azhar University, Cairo.

ABSTRACT

Bacillus methylotrophicus SCJ4, a marine bacteria isolated from highly polluted area of El-Max bay, Egypt showed a high potency for contemporaneous production of alkaline protease and α-amylase enzymes. In the current study, Plackett-Burman factorial design was applied to evaluate culture conditions affecting the production of both enzymes. Analysis of Plackett-Burman design results revealed that, the most significant variables affecting alkaline protease production were starch, K_2HPO_4, $MgSO_4$ and $CaCl_2$ with more than 6 folds increase in enzyme activity than basal medium (1185 $Uml^{-1}min^{-1}$ after 48h), while α-amylase production, were glucose, starch, peptone and $MgSO_4$ with 9 folds increase in enzyme activity than basal medium (280 $Uml^{-1}min^{-1}$after 48h).

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

Corresponding authors

ISSN: 0975-8585

INTRODUCTION

The increase of commercial demands for protease and amylase enzymes 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 [2,3,4]. The two enzymes are involved in many industrial applications such as pharmaceutical agents, food products, and laundry detergents [2,5,6,7]. Despite their wide spread in almost all living organisms, including plants, animals, and microorganisms, the special characteristics of microbial enzymes generally meet industrial demands [8,9,10]. 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[11] 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 [12]. 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 [13] and consequently does not guarantee getting the optimal conditions since it neglects interaction between variables [14]. Statistical-mathematical designs such as Plackett–Burman design present a more balanced alternative to the one-factor-at-a-time approach to fermentation improvement [15,16,17,18]. 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 [19]. 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 [20-24].

Bacillus methylotrophicus species is well known for its ability to assimilate methanol as a sole carbon source, hence its name [25] 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, and α-amylase by the marine *Bacillus methylotrophicus* SCJ4 locally isolated from El-Max bay of Alexandria, Egypt.

MATERIALS AND METHODS

Microorganism isolation, identification and cultivation conditions

Bacterial strain used in the current study, *Bacillus methylotrophicus* SCJ4, was isolated and identified through our previous work conducted on sediment and sea water samples collected from the El-Max bay [26]. 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 and α-amylase production were carried out in 250ml Erlenmeyer flasks containing 50ml liquid artificial seawater medium contained (g/l): NaCl, 27; $MgSO_4.7H_2O$, 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 %: $FeCl_3.4H_2O$, 0.24; EDTA, 14.6) and 1ml of trace metal solution (mg %: H_3BO_3, 60.0; $MnCl_2.H_2O$, 40.0; $(NH_4)6Mo_7O_{24}.4H_2O$, 37.0; $CuCl_2.2H_2O$, 4.0; $ZnCl_2$, 4.0; $CoCl_2.6H_2O$, 1.5). In screening for alkaline protease and α-amylase activity, the artificial sea water medium supplemented with (gelatin or starch) 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 Anson [27] 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.

Quantitative determination of α-amylase activity

Determination of α-amylase activity was carried out based on Fuwa's colorimetric method [28] of iodine starch color reaction with slight modification, where 50μL of diluted enzyme in 50mM phosphate buffer pH 7.0 was mixed with 100μL of 1.1% of the pre-warmed soluble starch in the same buffer and the reaction mixture was incubated at 50ºC for 10min. After incubation the reaction was stopped by adding 250μL from stopping solution (0.5N acetic acid and 0.5N HCl, prepared with ratio 5:1), then 100μL from the reaction mixed with 1ml iodine reagent (0.01% I_2 dissolved in 0.1% KI). The reduction in color was measured at 660 nm against control which prepared by the same method except that the stopping solution added before addition of the enzyme. One unit of amylase activity was defined as the amount of enzyme decreased the absorbance of 660 nm by 0.1 in 10 min.

Basal medium for enzymes production

For selecting a basal medium for enzymes production, different pre-optimized alkaline protease and α-amylase production media were initially tested for their ability to support both alkaline protease and α-amylase production, the media with the following composition (g/l) were used: Medium coded M1: Casamino acid, 10; glucose, 10; K_2HPO_4, 3; KH_2PO_4, 2; Na_2SO_4, 2; and $MgSO_4$, 0.1[29]. Medium coded M2: Glucose, 2; starch, 2; yeast extract, 10; peptone, 20; KH_2PO_4, 1; and $MgSO_4$, 0.1[30]. Medium coded M3: Glucose, 5; soybean meal, 10; K_2HPO_4, 3; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; and $CaCl_2.2H_2O$, 0.5 [31]. Medium coded M4: Citric acid, 10; $NaNO_3$, 10; K_2HPO_4, 5, $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.2; NaCl, 5.0 [8]. Medium coded M5: Starch, 10; peptone, 10; yeast extract, 20; KH_2PO_4, 0.05; $MnCl_2.4H_2O$, 0.015; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05; and $FeSO_4.7H_2O$, 0.01[32]. 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

Table 2: Plackett-Burman experiment 15 variables and its tested levels.

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

* Medium components expressed as (%) means gram%, inoculum size % means ml%.

ISSN: 0975-8585

Table 3: Randomized Plackett-Burman experimental design for evaluating factors influencing alkaline protease and α-amylase production from *Bacillus methylotrophicus* SCJ4.

Trial	Glucose	Starch	Soybean flour	Peptone	Yeast extract	K_2HPO_4	$MgSO_4$	NaCl	$CaCl_2$	$FeSO_4$	pH	Temperature	Incubation time	Inoculum size	Inoculum age	Protease activity $Uml^{-1}min^{-1}$	Amylase activity $Uml^{-1}min^{-1}$
1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	420.6	119.34
2	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	490.8	42.20
3	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	262.2	119.5
4	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	548.4	143.1
5	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	253.8	25.30
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	+1	532.8	73.00
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	+1	187.2	55.84
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	+1	694.2	66.64
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1	585.6	206.4
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	+1	198.6	77.10
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	-1	103.2	56.80
12	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	160.2	84.70
13	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	555.6	95.00
14	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	428.4	118.7
15	+1	-1	+1	-1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	496.0	100.0
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	107.4	74.30
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	399.6	87.80
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	374.2	89.58
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	360.8	87.30

ISSN: 0975-8585

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 methylotrophicus* 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:

$$Y = \beta_0 + \sum \beta_i x_i \qquad (1)$$

In this model Y representing the response (alkaline protease or α-amylase activity), β_0 is the model intercept, β_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.

RESULTS AND DISCUSSION

Microorganism isolation, identification and characterization

Table 1: 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	50ºC
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
Inositol fermentation	-ve
Sorbitol fermentation	-ve
Rhamnose fermentation	-ve
Sucrose fermentation	+ve
Lactose fermentation	-ve
Arabinose fermentation	+ve
Adonitol fermentation	-ve
Raffinose fermentation	-ve
Salicin fermentation	-ve

*ONPG=Hydrolysis of o-nitrophenyl-ß-d-galactopyranoside (ONPG) by action of ß- galactosidase
VP test = Voges-Proskauer test.

ISSN: 0975-8585

In the course of a qualitative screening program for alkaline protease and α-amylase activity, sixty seven isolates from highly xenobiotic contaminant area of El-Max bay, Egypt [26] were tested. From these isolates 24 (35.83%) showed alkaline protease activity, where 6 isolates (8.96%) showed α-amylase activity and 19 isolates (28.36%) showed contemporaneous activity for alkaline protease and α-amylase. Based on quantitative determination of protease and amylase activity, the isolate coded SCJ4 which was isolated from sea water sediments showed the highest potential for alkaline protease and amylase production in artificial sea water (186.4 $Uml^{-1}min^{-1}$) and (31.5 $Uml^{-1}min^{-1}$) under assay conditions, respectively. The morphological and physiological characteristics of the selected isolate coded SCJ4 are presented in **Table 1** and showed that it belongs to the genus *Bacillus* and has the ability to utilize methanol and ethanol as sole carbon sources. Molecular identification using 16S rRNA gene was conducted following gene isolation, sequencing, and deposition in GenBank under the accession number KF217257 [26]. The sequence analysis revealed a close relationship to *Bacillus methylotrophicus* L02 (Accession No. JN700125.1) with maximum identity (99%).

Different media used for testing protease and amylase 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 and amylase media were initially tested. Though, all tested media supported both of growth and 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 [34,35,36]. In contradiction, other studies reported a high protease yield in presence of ammonium sulfate, potassium nitrate or sodium nitrate as nitrogen source [8,37]. The highest alkaline protease production (347.1 $Uml^{-1}min^{-1}$) after 48h was obtained in M3 medium. On the other hand, the highest amylase activity (48.4 $Uml^{-1}min^{-1}$after 48h) was measured in M5 medium.

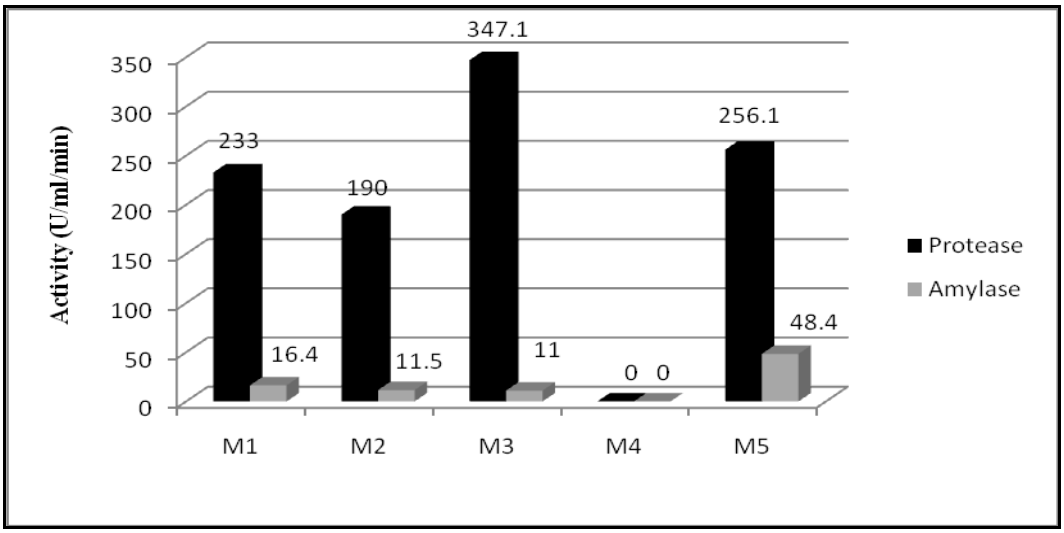

Figure 1. Effect of different pre-optimized medium on the alkaline protease and α-amylase production ($Uml^{-1}min^{-1}$)

In brief the co-production of both enzymes by *Bacillus methylotrophicus* SCJ4 was achieved in all test media except the medium with well defined components (M4). The production of both enzymes is not concomitant. A high titer of protease enzyme was recorded in M3, however a high titer of amylase was attained in M5. Therefore it is necessary to optimize the production of each enzyme individually using M3 as basal medium for protease enzyme and M5 basal medium for amylase enzyme in the subsequent optimization steps.

Evaluation of the factors influencing alkaline protease production

For alkaline protease, the data in **Table (3)** revealed a wide variation from 103.2 to 694.2 $Uml^{-1}min^{-1}$of 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: glucose, starch, soybean flour,

ISSN: 0975-8585

K_2HPO_4, $MgSO_4$, NaCl, $CaCl_2$, culture pH, cultivation temperature and cultivation time showed positive effect on alkaline protease activity, where peptone, yeast extract, $FeSO_4$, 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.

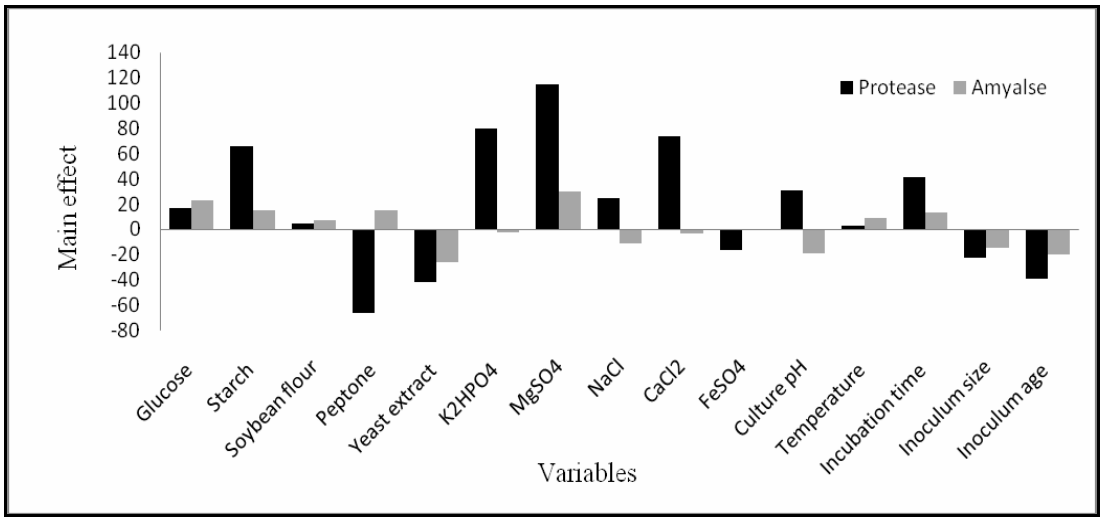

Figure 2. Effect of culture conditions on alkaline protease and α-amylase enzymes produced by *Bacillus methylotrophicus* SCJ4 based on Plackett-Burman design results.

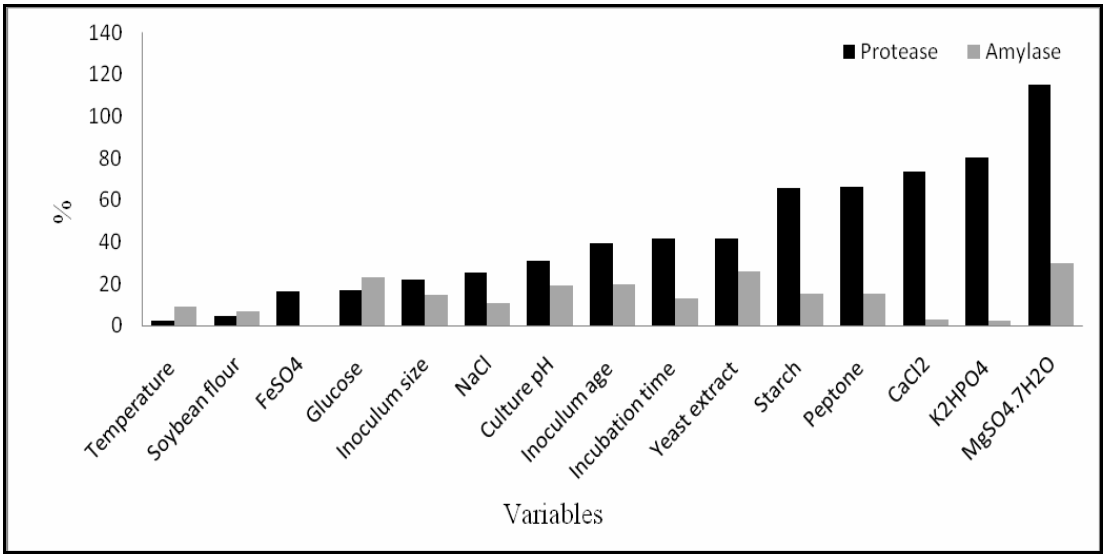

Figure 3.. Pareto plot for Plackett-Burman parameter estimates of alkaline protease and α-amylase enzymes produced by *Bacillus methylotrophicus* SCJ4.

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, $FeSO_4$ suppressed the enzyme production. It was reported that Mg^{2+}, Ca^{2+} and K^+ salts induce protease production and increase its stability [38,39].

ISSN: 0975-8585

Table 4: Statistical analysis of Plackett–Burman design showing coefficient, *t*-test values, *P*-values and confidence level (%) for variables affecting alkaline protease and α-amylase activities.

Variables	Protease enzyme				Amylase enzyme			
	Coefficient	*t*-test	*P*-value	Confidence level (%)	Coefficient	*t*-test	*P*-value	Confidence level (%)
Glucose	16.80	3.03	0.0564	94.36	22.84	23.52	0.0002	99.98
Starch	65.62	11.83	0.0013	99.87	15.15	15.60	0.0006	99.94
Soybean flour	4.30	0.77	0.4958	50.42	6.88	7.08	0.0058	99.42
Peptone	-65.96	-11.89	0.0013	99.87	15.46	15.92	0.0005	99.95
Yeast extract	-41.69	-7.52	0.0049	99.51	-26.13	-26.90	0.0001	99.99
K_2HPO_4	80.04	14.43	0.0007	99.93	-2.29	-2.35	0.1001	90.00
$MgSO_4.7H_2O$	114.75	20.69	0.0002	99.98	29.77	30.65	7.63E-05	99.99
NaCl	24.97	4.50	0.0205	97.95	-10.76	-11.08	0.0016	99.84
$CaCl_2$	73.27	13.21	0.0009	99.91	-3.26	-3.35	0.0440	95.60
$FeSO_4$	-16.02	-2.89	0.0631	93.69	0.19	0.19	0.8591	14.09
Culture pH	30.79	5.55	0.0115	89.85	-19.02	-19.59	0.0003	99.97
Temperature	2.50	0.45	0.6826	31.74	9.30	9.58	0.0024	99.76
Incubation time	41.22	7.43	0.0050	99.50	13.13	13.52	0.0009	99.91
Inoculum size	-22.10	-3.98	0.0283	97.17	-14.82	-15.26	0.0006	99.94
Inoculum age	-39.30	-7.09	0.0058	99.42	-19.61	-20.20	0.0003	99.97

In this study, carbon sources, starch, glucose 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 starch [35], and glucose [31,40]. Contrary to many finding stated, the high carbohydrates concentration, especially simple sugars, could suppress protease production [30,41,42], 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 [43,44]. Soybean flour is an agricultural waste with protein concentration ranged 51.2–53.2% [43]; 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 medium[45]. On the other hand [46-49], reported that the usage of peptone and yeast extract as a nitrogen sources maximize the enzyme production.

According to Plackett-Burman design results, a medium with the following composition (w/v%): Glucose, 1; starch, 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 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 $Uml^{-1}min^{-1}$). The second formula, without peptone, showed activity of (940.5 $Uml^{-1}min^{-1}$), where formula without peptone and yeast extract was the most potent one with activity of (1185 $Uml^{-1}min^{-1}$). According to these results, a medium of the following composition is expected to be optimum (g/l): starch, 10; soybean flour, 10; K_2HPO_4, 3; $MgSO_4$, 0.5; NaCl, 7; $CaCl_2$, 0.5; inoculum size of 1% from 12 h age cells, where maximum enzyme activity was 1185 $Uml^{-1}min^{-1}$after 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.

Evaluation of the factors affecting α-amylase production

Amylase activity analysis reflected a wide variation in activity from 42.2 to 206.4 $Uml^{-1}min^{-1}$ as shown in **Table (3)**. This variation reflected the importance of medium optimization in α-amylase production from *Bacillus methylotrophicus* SCJ4. The main effects of the examined factors on α-amylase activity were calculated and presented graphically in **Figure (2)**. Based upon the regression coefficients analysis of tested variables: glucose, starch, soybean flour, peptone, $MgSO_4$, cultivation temperature and time, showed positive effect on α-amylase activity where, yeast extract, K_2HPO_4, NaCl, $CaCl_2$, $FeSO_4$, culture pH, inoculum size and inoculum age were contributed negatively. **Figure (3)** shows the ranking of factor estimates in a Pareto chart.

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

$$Y_{activity} = 90.66 + 22.84X_1 + 15.15X_2 + 6.88X_3 + 15.46X_4 - 26.13X_5 + 2.29X_6 + 29.77X_7 - 10.76X_8 + 3.26X_9 + 0.019X_{10} + 19.02X_{11} + 9.3X_{12} + 13.13X_{13} - 14.82X_{14} - 19.61X_{15}$$

From the confidence level of the variables, it was apparent that variables namely, glucose, starch, peptone and $MgSO_4$ were the most significant variables affecting positively the α-amylase production. The p-values showed that, $MgSO_4$ was the most significant variable affecting amylase production; this finding is in accordance with studies reported using of metal ions as medium ingredients supported good growth and also influence higher α-amylase production [50]. The high significant effect of carbon source: glucose and starch in this experiment, is in accordance with reports indicated induction of α-amylase in presence of starch or its hydrolytic product [51,52]. On the other hand, yeast extract, culture pH, inoculum size and inoculum age, showed an adverse effect on enzyme production. Yeast extract, showed the highest negative effect, could be completely removed from medium compositions. Among all tested variables, K_2HPO_4, $CaCl_2$ and $FeSO_4$, showed little or non-significant effect on enzyme production. In contrary to most α-amylases which are well known to be metalloenzymes and Ca^{+2} ions are essential for maintaining the final structure of the enzyme [23], the α-amylase from *Bacillus methylotrophicus* EGY-SCJ4 is calcium-independent enzyme.

According to these results, a medium of the following composition is expected to be near optimum (g/l): glucose, 10; Starch, 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 $Uml^{-1}min^{-1}$ 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 co-production of alkaline protease and α-amylase by *Bacillus methylotrophicus* EGY-SCJ4. This is considered the first reported study focused on optimization the production of these enzymes by this isolate using experimental design. The variables are highlighted, and the results explained enzymes production are not concomitants. Finally, the enzyme yields were improved and reached to 6.4 and 8.9-folds compared to the basal medium for alkaline protease and α-amylase, respectively.

ACKNOWLEDGEMENT

Authors would like to acknowledge ULIXES project for funding the present work. Also, authors would like to thank Prof. Ranya A. Amer for molecular identification of the isolate and depositing the sequence in GenBank.

REFERENCES

[1] Hmidet N, Bayoudh A, Berrin JG, Kanoun S, Juge N, Nasri M. Process Biochem 2008; 43(5): 499–510.
[2] Kiran KK, Chandra TS. Appl Microbiol Biotechnol 2008; 77(5): 1023–1031.
[3] Merheb CW, Cabral H, Gomes E, Da-Silva R. Food Chem 2007; 104(1): 127–131.
[4] Abidi F, Liman F, Negib MM. Process Biochem 2008; 43(9): 1202–1208.
[5] Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Process Biochem 2002; 38(11): 1599–1616.
[6] Hadj-Ali NE, Agrebi R, Ghorbel-Frikha B, Sellami-Kamoun A, Kanoun S, Nasri M. Enzyme Microb Technol 2007; 40(4): 515–523.
[7] Sellami-Kamoun A, Haddar A, Hadj-Ali NE, Ghorbel-Frikha B, Kanoun S, Nasri M. Microbiol Res 2008; 163(3): 299–306.
[8] Johnvesly B, Naik GR. Process Biochem 2001; 37(2): 139–144.
[9] van der Maarel MJ, van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L. J Biotechnol 2002; 94(2):137–155.
[10] Aquino AC, Jorge JA, Terenzi HF, Polizeli ML. Appl Microbiol Biotechnol 2003; 61(4): 323–328.
[11] Joo HS, Chang CS. Process Biochem 2005; 40(3-4): 1263–1270.
[12] Chandrasekaran M. J Mar Biotechnol 1997; 5(2/3):86–89.
[13] Adinarayana K, Ellaiah P, Srinivasulu B, Bhavani Devi R, Adinarayana G. Process Biochem 2003; 38(11): 1565–1572.
[14] Myers, R.H., Montgomery, D.C. Response surface methodology: process and product optimization using designed experiments. New York: Wiley, 2002.
[15] Box GEP, Behnken DW. Technometrics 1960; 2(4): 455–475.
[16] Box GEP, Hunter WG, Hunter JS. Statistics for Experimenters. John Wiley & Sons, Inc., New York, N.Y. 1978.
[17] Krishnan S, Prapulla SG, Rajalakshmi D, Misra MC, Karanth NG. Bioprocess Eng 1998; 19(1): 61–65.
[18] El-Helow ER, Abdel-Fattah YR, Ghanem KM, Mohamed EA. Appl Microbiol Biotechnol 2000; 54(4): 515–520.
[19] Plackett RL, Burman JP. Biometrika 1946; 33(4): 305–325.
[20] Pranaw K, Singh S, Dutta D, Chaudhuri S, Ganguly S, Nain L. Biotechnol. Res. Int. 2014. Article ID 293434, 11 pages.
[21] Swetha S, Varma A, Padmavathi T. 3 Biotech. 2014; 4(4): 439–445.
[22] Nassar FR, Abdelhafez AA, El-Tayeb TS, Abu-Hussein SH. Br Microbiol Res J 2015; 6(5): 255–269.
[23] Prajapati VS, Trivedi UB, Patel KC. 3 Biotech. 2015; 5(2): 211–220.
[24] Chandra SJ, Pallavi P, Anuradha BS, Reddy SR. Afr J Microbiol Res 2015; 9(28): 1738–1744.
[25] Madhaiyan M, Poonguzhali S, Kwon SW, Sa TM. Int J Syst Evol Microbiol 2010; 60(10): 2490–2495.
[26] Amer RA, Abdel-Fattah YR. JOKULL 2014; 64(4): 223–249.
[27] Anson ML. J Gen Physiol 1938; 22(1): 79–89.
[28] Fuwa HA. J Biochem 1954; 41(5): 583–603.
[29] Beg QK, Sahai V, Gupta R. Process Biochem 2003; 39(2): 203–209.
[30] Abdel-Fattah YR, El-Enshasy HA, Soliman NA, El-Gendi H. J. Microbiol. Biotechnol. 2009; 19(4): 378–386.
[31] Nadeem M, Qazi JI, Baig S, Syed Q. Food Technol Biotechnol 2008; 46(4): 388–394.
[32] Ashwini K, Kumar G, Loganathan K, Rao KVB. Arch Appl Sci Res 2011; 3(1): 33–42.
[33] Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. Biotechnol Appl Biochem 2000; 31(2): 135–152.
[34] Ferrero AM, Carto GR, Abate CM, Baigori MD, Sineriz F. Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: Appl Microbiol Biotechnol 1996; 45(3): 327–332.
[35] Chauhan B, Gupta R. Process Biochem 2004; 39(12): 2115–2122.
[36] Reddy LVA, Wee YJ, Yun JS, Ryu HW. Bioresour Technol 2008; 99(7): 2242–2249.
[37] Sinha N, Satyanarayana T. Indian J Microbiol 1991; 31(4): 425–430.
[38] Ellaiah P, Srinivasulu B, Adinarayana K. J Sci Ind Res 2002; 61(9): 690–704.

[39] Thumar JT, Singh SP. Braz J Microbiol 2007; 38(4): 766–772.
[40] El Enshasy H, Abuoul-Enein A, Helmy S, El Azaly Y. Aust J Basic and Appl Sci 2008; 2(3): 583–593.
[41] Patel RK, Dodia MS, Singh SP. Process Biochem. 2005; 40(11): 3569–3575.
[42] Falahatpishe H, Jalali M, Badami N, Mardani N, Khosravi-Darani K. Iran J Biotech 2007; 5(2): 110–113.
[43] Silva GMM, Bezerra RP, Teixeira JA, Porto TS, Lima-Filho JL, Porto ALF. Electron J Biotechnol 2015; 18(1): 16–19.
[44] Basu MJ, Indra V, Hemapriya J, Vijayanand S. Int J Curr Res Aca Rev 2015; 3(4): 309–315.
[45] Frankena J, Koningstein GM, van Verseveld HW, Stouthamer AH. Appl Microbiol Biotechnol 1986; 24(2): 106–112.
[46] Puri S, Beg QK, Gupta R. Curr Microbiol 2002; 44(4): 286–290.
[47] Joo HS, Kumar CG, Park GC, Paik SR, Chang CS. J Appl Microbiol 2003; 95(2): 267–272.
[48] Mehta VJ, Thumar JT, Singh SP. Bioresource Technol 2006; 97(14): 1650–1654.
[49] Patel RK, Dodia MS, Joshi RH, Singh SP. World J Microbiol Biotechnol 2006; 22(4): 375–382.
[50] Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey A. Food Technol Biotechnol 2006; 44(2): 173–184.
[51] Lachmund A, Urmann U, Minol K, Wirsel S, Ruttkowski E. Curr Microbiol 1993; 26(1): 47–51.
[52] Morkeberg R, Carlsen M, Neilsen J. Microbiology 1995; 141(10): 2449–2454.