World Journal of Agricultural Sciences 5 (3): 375-383, 2009
ISSN 1817-3047
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Optimization of Thermophilic Protease Production in Bacillus Mixed Cultures under Mesophilic Conditions

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Abstract: Proteases execute a large variety of functions and have important biotechnological applications. They could be useful for degradation of different protein sources, so find potential application for waste treatment, bioremediation processes, in detergents and in leather industry. In this work, five different Bacillus species were used (*Bacillus subtilis*, *Bacillus pumilus, Bacillus therigienesis, Bacillus licheniformis* and *Geobacillus sp.)*. These species were detected for their thermophilic protease. The main aim of this study is to optimize the production of the thermophilic protease under mesophilic condition and different nutritional factors by using experimental design represented in Plackett-Burman to randomize nine media constituents and five Bacillus strains. The maximum protease activity in medium having high level(+1) of trypton soya bean, glucose, yeast extract, FeSO4, trace element solution and four Bacillus strains (*Bacillus subtilis*, *Bacillus therigienesis, Bacillus licheniformis* and *Geobacillus sp.)* was 626.32 Units/ml. The statistical data of the overall experiments proved that glucose, KH_2PO_4, yeast extract and *Geobacillus sp.* are the most effective variables with confidence level percentage equal to 71.83, 82.73, 83.19 and 85.84% respectively. The results proved also that the applied methods and the use of mixed Bacillus culture were the most effective. So, we recommends the use of the strategies included in this study for further applications concerning thermophilic protease production using mixed cultures under mesophilic condition and different nutritional factors in industrial scales to decrease the cost and increase the profit.

Key words: Thermophilic proteases · Bacillus · Plackett-Burman · Mesophilic condition

INTRODUCTION

Among the various proteases, bacterial protease was the most significant compared with animal, fungi and plant protease. Bacillus species were specific producers of extracellular protease. For example *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* were the most known species used in industry for alkaline protease production [1]. In nature, protease have valuable biochemical and physiological functions. They cleave proteins into amino acids or short peptides. Some proteases can cleave more specifically to produce useful peptides. They were represent one of the three largest groups of industrial enzymes. They were find applications in detergents, wool quality improvement, meat tenderization, leather, food, pharmaceutical industry, waste processing industries etc, [2-8].

Different Bacillus species were known for their ability to produce thermostable proteases that have an optimum activity more than 85-90°C [9,10].

Thermostable enzymes can be produced under mesophilic condition. In industrial scale, where cost and profit govern the production processes, mesophilic condition has more opportunity while it requires less energy [11]. The demand on the thermostable protease increases due to its importance in some applications.

To exceed the thermophilic protease production, we were used different nutritional factors and bacillus mixed

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cultures. Plackett-Burman design was used for the optimization of different nutritional conditions for thermophilic protease production [11,12]. The results were analyzed statistically using different statistical methods [4,13]. The amount of thermophilic protease has been increased significantly.

MATERIALS AND METHODS

Microorganism: Five Bacillus species were isolated from the Egyptian ecosystem and identified previously as *Bacillus subtilius*, *Bacillus pumilus*, *Bacillus therigienesis*, *Bacillus licheniformis* and *Geobacillus*. The strains were selected out of many strains for their proteolytic activity. They grow routinely in LB medium (Luria-Bertani) at 37°C and maintained at -70°C by adding 300 μl glycerol to each 1 ml culture in suitable plastic container [14].

Detection of the Proteolytic Activities on Plates: The different Bacillus strains were screened for their ability to produce protease enzymes using agar well diffusion technique. 1 gm gelatin was suspended in 100 ml water and autoclaved. After autoclaving, the soluble components were added to sterile water agar (16 g agar/l). The suspension then stirred gently and distributed in Petri dishes (25 ml/plate). After complete solidification of the agar on plates, wells were punched out of the agar, by using a clean sterile cork borer. The base of each hole was sealed with drop of a melted sterile water agar (15 g agar per liter H_2O) using sterile Pasteur pipette. The different bacillus species were cultivated on skim milk broth media [2]. Seventy five-μl of the cell free supernatant of each strain was added to each well, then the plates were incubated at -4°C for 30 min and then incubated overnight at 60°C [2].

Visualization of the Proteases Clear Zone Method: Extracellular protease activity on the plates were detected according to amido black staining the method [15]. Staining was performed with 0.1% amido black in methanol-acetic acid-water 30:10:60 (v/v/v) for 5-20 minutes [15].

Thermophilic Proteases Production Media: The following cultivation media as well as five microbial strains were used for the production of thermophilic Protease: Trypton Soya been; Skim milk; Lactose; Glucose; Yeast extract; KH_2PO_4; $FeSO_4$ solution; Trace elements solution and KCl_2. Different media constituents and the Bacillus strains have been used in amount represent +1 and -1 according to Plackett-Burman in quantities as in Table 1. The Trace elements solution consists of: $ZnSO_4$. 7 H_2O 10 mg/l; $MnCl_2$. 4 H_2O 3 mg/l; H_3BO_3 30 mg/l; $CoCl_2$. 6 H_2O 20 mg/l; $CuCl_2$. 2 H_2O 1 mg/l; $NiCl_2$. 6 H_2O 2 mg/l and Na_2MoO_4. 2 H_2O 3 mg/l. The trace elements solution and $FeSO_4$ solution (12 mg/l) were sterilized each with 0.22 μm sterilize filter system. In all the experiments, the pH of the medium was adjusted at 7. The different media constituents and the Bacillus strains have been used in amount represent +1 and -1 according to Plackett-Burman as in Table 1.

Table 1: Different media constituents as used in Plackett-Burman [+1, -1]

Variables Name	+1	-1	Unit/100 ml
B. subtilis	100.00	10.00	μl
B. pumilus	100.00	10.00	μl
B. therigienesis	100.00	10.00	μl
B. licheniformis	100.00	10.00	μl
Geobacillus sp.	100.00	10.00	μl
Trypton Soya been	10.00	5.00	mg
Skim milk	10.00	0.20	mg
Lactose	10.00	0.40	gm
Glucose	2.00	0.40	gm
Yeast extract	0.40	0.08	gm
KH_2PO_4	0.10	0.01	gm
$FeSO_4$ solution	5.00	1.00	μl
Trace elements solution	5.00	1.00	μl
KCl_2	0.30	0.03	gm

Preparation of Equal Inocula: Different Bacillus species were pre-cultivated on LB agar plate overnight at 37°C. One loop from the fresh colonies from each specie was taken and inoculated in test tube contain LB broth media. The tubes incubated overnight in shaker incubator at 200 rpm, pH 7 and 37°C. The OD_{600} for each species has been adjusted to 0.025 nm by adding sterilized water to the culture. Different volumes have been used to inoculate the 100 ml media as in Table 1.

In Flask Fermentation Conditions: Protease production was conducted using 250 ml Erlenmeyer-Flask containing 100 ml media, the shaking rate was 200 rpm at 37°C. Media compositions were changed when running Plackett-Burman different experiments. The amount of each medium constituent has been randomized as described in Tables 1 and 2.

Table 2: Plackett-Burman experiments (Plackett and Burman 1946).

B. subtilius	*B. pumilus*	*B. therigienesis*	*B. lichenifermis*	*Geobacillus sp*	Trypton Soya bean	Skim milk	Lactose	Glucose
1 (100)	1 (100)	1(100)	1 (100)	-1 (10)	-1 (5)	1 (10)	1 (2)	-1 (0.4)
1 (100)	-1 (10)	-1 (10)	1 (100)	1 (100)	-1 (5)	1 (10)	1 (2)	-1 (0.4)
1 (100)	-1 (10)	1 (100)	1 (100)	1 (100)	1 (10)	-1 (0.2)	-1 (0.4)	1 (2)
1 (100)	1 (100)	1 (100)	-1 (10)	-1 (10)	1 (10)	1 (10)	-1 (0.4)	1 (2)
1 (100)	1 (100)	-1 (10)	-1 (10)	-1 (10)	-1 (5)	1 (10)	-1 (0.4)	1 (2)
-1 (10)	1 (100)	-1 (10)	1 (100)	-1 (10)	1 (10)	1 (10)	1 (2)	1 (2)
1 (100)	1 (100)	-1 (10)	-1 (10)	1 (100)	1 (10)	-1 (0.2)	1 (2)	1 (2)
1 (100)	1 (100)	-1 (10)	1 (100)	1 (100)	-1 (5)	-1 (0.2)	-1 (0.4)	-1 (0.4)
-1 (10)	1 (100)	1 (100)	1 (100)	1 (100)	-1 (5)	-1 (0.2)	1 (2)	1 (2)
-1 (10)	1 (100)	-1 (10)	1 (100)	1 (100)	1 (10)	1 (10)	-1 (0.4)	-1 (0.4)
-1 (10)	-1 (10)	-1 (10)	1 (100)	-1 (10)	1 (10)	-1 (0.2)	1 (2)	1 (2)
-1 (10)	-1 (10)	-1 (10)	-1 (10)	-1 (10)	-1 (5)	-1 (0.2)	-1 (0.4)	-1 (0.4)
-1 (10)	1 (100)	1 (100)	-1 (10)	1 (100)	1 (10)	-1 (0.2)	-1 (0.4)	-1 (0.4)
-1 (10)	-1 (10)	-1 (10)	-1 (10)	1 (100)	-1 (5)	1 (10)	-1 (0.4)	1 (2)
1 (100)	-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (5)	-1 (0.2)	-1 (0.4)	1 (2)
-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (10)	-1 (5)	-1 (0.2)	1 (2)	-1 (0.4)
1 (100)	-1 (10)	-1 (10)	-1 (10)	-1 (10)	1 (10)	-1 (0.2)	1 (2)	-1 (0.4)
-1 (10)	-1 (10)	1 (100)	-1 (10)	1 (100)	-1 (5)	1 (10)	1 (2)	1 (2)
1(100)	-1 (10)	1 (100)	-1 (10)	1 (100)	1 (10)	1 (10)	1 (2)	-1 (0.4)
-1 (10)	-1 (10)	1 (100)	1 (100)	-1 (10)	1 (10)	1 (10)	-1 (0.4)	-1 (0.4)

B. subtilius	*B. pumilus*	*B. therigienesis*	*B. lichenifermis*	*Geobacillus sp*	Yeast extract	KH_2PO_4	$FeSO_4$ solution	Trace elements solution	KCl_2
1 (100)	1 (100)	1(100)	1 (100)	-1 (10)	1 (0.4)	1 (0.1)	-1 (1)	-1 (1)	-1 (0.01)
1 (100)	-1 (10)	-1 (10)	1 (100)	1 (100)	-1 (0.08)	-1 (0.01)	-1 (1)	1 (5)	-1 (0.01)
1 (100)	-1 (10)	1 (100)	1 (100)	1 (100)	1 (0.4)	-1 (0.01)	1 (5)	1 (5)	-1 (0.01)
1 (100)	1 (100)	1 (100)	-1 (10)	-1 (10)	1 (0.4)	-1 (0.01)	-1 (1)	-1 (1)	-1 (0.01)
1 (100)	1 (100)	-1 (10)	-1 (10)	-1 (10)	-1 (0.08)	1 (0.1)	1 (5)	1 (5)	1 (0.3)
-1 (10)	1 (100)	-1 (10)	1 (100)	-1 (10)	-1 (0.08)	-1 (0.01)	1 (5)	1 (5)	-1 (0.01)
1 (100)	1 (100)	-1 (10)	-1 (10)	1 (100)	-1 (0.08)	-1 (0.01)	-1 (1)	-1 (1)	1 (0.3)
1 (100)	1 (100)	-1 (10)	1 (100)	1 (100)	1 (0.4)	-1 (0.01)	1 (5)	-1 (1)	1 (0.3)
-1 (10)	1 (100)	1 (100)	1 (100)	1 (100)	-1 (0.08)	1 (0.1)	1 (5)	-1 (1)	-1 (0.01)
-1 (10)	1 (100)	-1 (10)	1 (100)	1 (100)	1 (0.4)	1 (0.1)	-1 (1)	1 (5)	1 (0.3)
-1 (10)	-1 (10)	-1 (10)	1 (100)	-1 (10)	1 (0.4)	1 (0.1)	-1 (1)	-1 (1)	1 (0.3)
-1 (10)	-1 (10)	-1 (10)	-1 (10)	-1 (10)	-1 (0.08)	-1 (0.01)	-1 (1)	-1 (1)	-1 (0.01)
-1 (10)	1 (100)	1 (100)	-1 (10)	1 (100)	-1 (0.08)	1 (0.1)	-1 (1)	1 (5)	-1 (0.01)
-1 (10)	-1 (10)	-1 (10)	-1 (10)	1 (100)	1 (0.4)	1 (0.1)	1 (5)	-1 (1)	-1 (0.01)
1 (100)	-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (0.08)	1 (0.1)	-1 (1)	1 (5)	1 (0.3)
-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (10)	1 (0.4)	-1 (0.01)	1 (5)	1 (5)	1 (0.3)
1 (100)	-1 (10)	-1 (10)	-1 (10)	-1 (10)	1 (0.4)	1 (0.1)	1 (5)	1 (5)	-1 (0.01)
-1 (10)	-1 (10)	1 (100)	-1 (10)	1 (100)	1 (0.4)	-1 (0.01)	-1 (1)	1 (5)	1 (0.3)
1(100)	-1 (10)	1 (100)	-1 (10)	1 (100)	-1 (0.08)	1 (0.1)	1 (5)	-1 (1)	1 (0.3)
-1 (10)	-1 (10)	1 (100)	1 (100)	-1 (10)	-1 (0.08)	-1 (0.01)	1 (5)	-1 (1)	1 (0.3)

Preparation of L-tyrosine Standard Curve: 1.1-mM L-tyrosine was dissolved in 100 ml deionized water by heating gently (without boiling). After complete dissolving of the L-tyrosine, the standard curve has been generated [2] by using different concentrations of L-tyrosine.

Preparation of Casein-Universal Buffer: Universal buffer was prepared; 40 mM H_3PO_4, 40 mM acetic acid and 40 mM H_3BO_3 [16]. The pH was adjusted to 8 using 0.2 M NaOH. 0.325 mg casein (Protein Hydrolysate-Hy-Case®Amino-Fluka®), then dissolved in 50 ml of the Universal buffer. The mixture was dissolved by heating gently to 80-90°C without boiling. The mixture was used immediately or preserved in -20°C for further usage [2].

Protease Activity: 0.3 ml of each supernatant, which contain the crude enzymes was added to the same volume of the Casein-Universal buffer (pH 8). The enzyme-substrate mixture was incubated at 60°C for 30 min. After the incubation period, the enzyme reaction was stopped by adding 0.6 ml of 10%

Trichloro acetic acid. The mixture then was stand at room temperature for 15 min then centrifuged at 10^4 rpm for 10 min (Biofuge 15 - Heraeus Sepatech). The absorbance of each supernatant was determined spectrophotometrically at 280 nm (PerkinElmer-UV/VIS Spectrometer Lambda), its tyrosine content was derived from the tyrosine standard curve and each enzyme activity determined as Unit/ml. One unit of the enzyme activity was defined as the amount of enzyme which release 1 micro mol of tyrosin /min under the assay condition.

Experimental Designs

Plackett-Burman: The different variables were: five microbial strains represent *B. subtilius* (X_1), *B. pumilus* (X_2), *B. therigienesis* (X_3), *B. lichenifermis* (X_4) and *Geobacillus sp* (X_5) as well as nine media constituents represent: Trypton Soya been (X_6); Skim milk (X_7); lactose (X_8); Glucose (X_9); Yeast extract (X_{10}); KH_2PO_4 (X_{11}); Trace elements solution (X_{12}); $FeSO_4$ solution (X_{13}) and KCl_2 (X_{14}). The fourteen variables were randomized according to Plackett-Burman design as in Table 1 [13]. Twenty experiments following Plackett-Burman design have been conducted as in table 2 to optimize fourteen variables represented at two levels (high and low), which are donated by +1 and -1 as in table 2. All of the twenty experiments were conducted using 250 ml flasks containing 100 ml media at 37°C under shaking conditions (200 rpm for 48 h). The OD_{600} for each seeding strain was adjusted to 0.025 nm. All quantities were calculated as x/100 ml culture as in Table 1.

Multiple Regression Analysis of Plackett-Burman Experiments: The results of the Plackett-Burman design experiments were applied to linear multiple regression analysis using Microsoft Excel 2002 software. The linear multiple regression analysis was conducted for the thermophilic protease activity as response. From the statistical analysis of the data in table 2, which was summarized in tables 4 and 5, the variables whose confidence levels were bigger than or equal to 90% were considered to be significant for the protease production. Variables with confidence levels were less than 90% until 70% were considered as effective [17].

Generating 1st Order Model: The model created from the analysis of Plackett-Burman experimental design using liner multiple regression analysis is based on the 1st order-model

$$Y=\beta_0+\Sigma\beta_i X_i^{13}$$

Where Y is the predicted response, β_0 model constant, β_i variables linear coefficient.

ANOVA test was generated for each response to determine the relationship between the variables at the 90% or higher confidence level

RESULTS

Detection of the Thermophilic Proteolytic Activity on Plates: The thermophilic proteolytic activity of each strain was determined on gelatin agar plate. The clear zone of each well appears clearly after visualization using staining method as in Figure 1. This technique gives preliminary fast idea about the ability of different Bacillus strains to produce proteolytic enzymes. Activity of these enzymes appear as a clear zoon around the well, which can be used also to compare different samples as in Figure 1.

Plackett-Burman Main Effect: The experimental design using Plackett-Burman method was produced using +1 and -1 for each variable as in Table 2 where twenty experiment have been conducted. The results are summarized as thermophilic protease (Units/ml). The mean of +1 experiments have been calculated using the following formula: $(?+1)/ n_{(+1)}$. While the mean of -1 experiments have been calculated using the following formula: $(?-1)/ n_{(-1)}$. The main effect of each variable has been calculated from the following formula: Main effect = $?(+1)/n_{(+1)}-?(-1)/n_{(-1)}$. The different main effect of the different variables for thermophilic protease were calculated and summarized in Figure 2 and Table3.

Plackett-Burman Design: The multiple linear regression following model was performed as in table 4 to describe the relationship between the amount of thermophilic protease produced from Plackett-Burman randomization for 14 variables in twenty experiments as in Table 2.

Thermophilic protease = 314.686 + 13.277 * $FeSO_4$ solution+ 30.833 * Glucose - 13.232 * KCl_2 - 40.655 * KH_2PO_4 - 17.816 * Lactose + 10.535 * Skim milk + 7.262 * *Bacillus subtilius* - 19.63 * *Bacillus pumilus* + 18.677 * *Bacillus therigienesis* + 2.113 * *Bacillus lichenifermis* + 44.592 * *Geobacillus sp.* + 23.587 * Trace elements solution + 21.846 * Trypton Soya been + 41.184 * Yeast extract

Fig. 1: Different proteolytic activity on the Agar well diffusion method using Gelatin plates stained with amido black

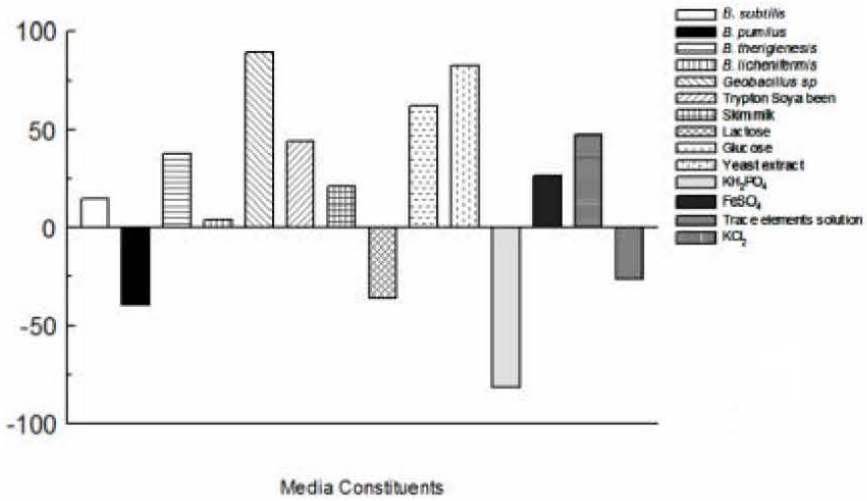

Fig. 2: Main effect on thermophilic protease amount of the media constituents after randomization using Plackett-Burman design

Table 3: Main effect $[\Sigma(+1)/n_{(+1)}-\Sigma(-1)/n_{(-1)}]$ of each variable using Plackett-Burman design on thermophilic Proteases

	Values		
Variables	$(\Sigma+1)/n_{(+1)}$	$(\Sigma-1)/n_{(-1)}$	Main effect $\Sigma(+1)/n_{(+1)}-\Sigma(-1)/n_{(-1)}$
B. subtilius	321.948	307.424	14.524
B. pumilus	295.056	334.316	-39.260
B. therigienesis	333.363	296.009	37.354
B. lichenifermis	316.799	312.573	4.226
Geobacillus sp	359.278	270.094	89.184
Trypton Soya been	336.532	292.840	43.692
Skim milk	325.221	304.151	21.070
Lactose	296.870	332.502	-35.632
Glucose	345.519	283.853	61.666
Yeast extract	355.870	273.502	82.368
KH_2PO_4	274.031	355.341	-81.310
$FeSO_4$ solution	327.963	301.409	26.554
Trace elements solution	338.273	291.099	47.174
KCl_2	301.454	327.918	-26.464

Table 4: Linear Multiple regression analysis of Plackett-Burman design [thermophilic proteases]

Variables	Estimate	Standard Error	T Statistic	P-Value	Confidence%
Constant	314.686	25.5653	12.309100	0.0001	99.99
$FeSO_4$ solution	13.277	25.5653	0.519337	0.6257	37.43
Glucose	30.833	25.5653	1.206050	0.2817	71.83
KCl_2_	-13.232	25.5653	-0.517580	0.6268	37.32
KH_2PO_4_	-40.655	25.5653	-1.590240	0.1727	82.73
Lactose	-17.816	25.5653	-0.696880	0.5169	48.31
Skim Milk	10.535	25.5653	0.412082	0.6974	30.26
Bacillus subtilius	7.262	25.5653	0.284057	0.7878	21.22
Bacillus pumilus	-19.630	25.5653	-0.767840	0.4773	52.27
Bacillus therigienesis	18.677	25.5653	0.730561	0.4978	50.22
Bacillus lichenifermis	2.113	25.5653	0.082651	0.9373	6.27
Geobacillussp.	44.592	25.5653	1.744240	0.1416	85.84
Trace elements solution	23.587	25.5653	0.922619	0.3985	60.15
Trypton soya been	21.846	25.5653	0.854519	0.4318	56.82
Yeast extract	41.184	25.5653	1.61094	0.1681	83.19

Table 5: ANOVA test for thermophilic protease response

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	177856.0	14	12704.0	0.97	0.5619
Residual	65358.3	5	13071.7		
Total (Corr.)	243214.0	19			

R-squared = 73.1273 percent R-squared (adjusted for d.f.) = 0.0 percent Standard Error of Est. = 114.331 Mean absolute error = 50.0864

Table 6: Residual output

Residual Output			
Observation	PredictedThermophilic activity Units/ml	Residuals	Standard Residuals
1	195.453	-69.353	-1.182474091
2	332.659	26.591	0.453378636
3	598.855	27.465	0.468280405
4	413.527	69.353	1.182474091
5	216.067	62.443	1.064658049
6	321.603	-45.783	-0.780603742
7	299.823	-86.013	-1.466528398
8	343.245	-10.805	-0.184226098
9	254.895	88.545	1.509699196
10	332.793	53.567	0.913321552
11	240.659	43.251	0.737432943
12	192.113	70.227	1.197375859
13	288.947	-53.567	-0.913321552
14	391.645	-105.205	-1.793753503
15	249.283	-62.443	-1.064658049
16	284.207	-8.387	-0.142999008
17	289.483	-8.273	-0.141055299
18	468.833	8.387	0.142999008
19	281.085	51.035	0.870150754
20	298.545	-51.035	-0.870150754

The P-value in the ANOVA in table 5 was greater or equal to 0.10. There was not a statistically significant relationship between the variables at the 90% or higher confidence level.

The R-Squared statistic indicates that the model as fitted explains 73.12% of the variability in Thermophilic protease amount. The standard error of the estimate was revealed the standard deviation of the residuals to be 114.331. The mean absolute error of 50.0864 was the average value of the residuals. The predicted thermophilic activity as Units/ml were summarized in table 6.

DISCUSSION

In the industrial applications, the use of the mixed culture will be decreased the cost and increased the profit, while different microbes with different genetic materials will be collaborated in utilizing particular substrates or group of substrates. Protease enzymes were the most important group of thermophilic technical enzymes. Investigators have been proved that many *Bacillus sp.* were good producers of extracellular protease at high temperature and this enzyme would be thermostable protease [18,19]. Performing production process with minimum requirements and optimum results were always an aim [8]. Media optimization has been showed an increasing interest for optimizing different products [20,21]. Experimental design has been used to optimize production process of protease [11,12]. Plackett-Burman was one of the most favourable experimental design methods and it was used usually in

the optimization of complicated process [22,23], by conducting randomization as in Table 2 using law and high levels of each variable. Plackett-Burman is based on randomization of the variables under investigation using minimum number of experiments [13]. Plackett-Burman was able to map the points of strength and weakness in any process which contains different and/or heterogenous variables. Bacillus species were widely used in protease production. Perhaps, the most important criteria in Bacillus strains were their ability to utilize cheap carbon sources. Thermophilic enzymes including proteases were the subject of an interest due to their unique properties. Economically, the production of thermophilic protease from mesophilic strains or using mesophilic condition was recommended [24,25]. The cost of running fermentation under mesophilic condition is rather cheap than that under thermophilic one. In this study, five thermophilic Bacillus species were cultivated under mesophilic condition and subjected to optimization by the use of Plackett-Burman experimental design to optimize two main groups of variables. The first group was the five Bacillus species were well known for their ability to produce protease enzyme [9, 26, 27]. The second group was the nine media constituents, which represent the nutritional factors. The fourteen variables were randomized using +1 and -1 as shown in table 2. The results were proved the importance of using Plackett-Burman experiments to randomize and optimize complicated variables [22,23]. The minimum yield was at experiment number 1 equal to 126.10 Units/ml while the maximum yield was at experiment number 3 and equal to 626.32 Units/ml.

By logical analysis of the difference between experiment number 1 & 3, it was found that in experiment no 3, skim milk, lactose, KH_2PO_4 and KCl_2 were in their low level -1, while Trypton, Glucose, yeast extract, $FeSO_4$ solution and Trace elements solution were in their high level +1. The neglection of the factors, which were the same in experiment number 1 and 3 [KCl_2 and yeast extract] will be lead to obtaining the variables that caused this significant increase in the protease amount: Trypton soya been (+1), Skim milk (-1), Lactose (-1), Glucose (+1), KH_2PO_4(-1), $FeSO_4$(+1) and Trace elements solution (-1). The same results will be obtained in case of compairing Bacillus different species. These species were *B. pumilus* (-1) and *Geobacillus sp.* (+1). They will be responsible for increasing the production of the therophilic prtotease.

The use of linear multiple regression analysis of Plackett Burman experiments was important to select the most effective variables which give the highest confidence level %. This will be lead to select each of glucose, KH_2PO_4 yeast extract and *Geobacillus sp* as the most effective variable where they have a Confidence level % higher than 70% [17].

These results will be agree with the logical analysis of the data. Only yeast extract was not detected by logical analysis of the data. The comparison between the experiment number 1 and 3 was resulted in deleting yeast extract from variables effect on the thermophilic protease production. This clearly put a conclusion that logical analysis of the data could be better in some cases than statistical one for selecting the most effective variables. The linear multiple regression analysis will be investigate the overall experiments, while logical analysis will be investigated the differences more closely. Meanwhile, the result was expected by using linear regression analysis, all the data will be included, while logical analysis investigate only the difference between experiments number 1 and 3, which gave the highest and lowest activities.

In this study, optimization of the environmental conditions has been neglected such as temperature, pH, shaking rate etc. and should be considered in the further optimization studies. In conclusion, this study showed the opportunity to produce and optimize the thermophilic protease under mesophilic condition using mixed culture and Plackett-Burman design. We recommended that the production of thermophilic protease under mesophilic condition using wild type of Bacillus culture, strategies and conditions used in this study. This can be achived by reducing the production cost, increasing the products quantity and the overall profits. Protease applications ranged from technical quality for industrial, commercial use and bioremediation prosses to high quality in case of products involved un pharmaceutical and medicinal applications [2-7].

ACKNOWLEDGMENT

We acknowledge the grant provided from the Arab Science and Technology Foundation, which support this study [14-08-MS0810257-ALJ].

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