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Scientific Research

Optimization of Protease Production by *Bacillus mojavensis* A21 on Chickpea and Faba Bean

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Abstract

Response surface methodology (RSM) was employed to optimize the medium composition and culture conditions for the production of alkaline proteases by *Bacillus mojavensis* A21 on uncommon substrates: chickpea (CF) and faba bean (FF) flours. A significant positive influence of temperature, CF, FF, incubation time and inoculums size on the protease production was evaluated by Plackett Burman Design. Among these, CF was the most influential factor. The enhancement of protease to 9127 U/ml was achieved with the optimization procedure on the medium composed of (g/l): CF, 40; FF 30, NaCl 2.0; KH_2PO_4 1; K_2HPO_4 1; $CaCl_2$, 0.1; $MgSO_4$ 0.1. The cultures were conducted for 72 hours with an IS of 2%, at 30˚C, an agitation speed of 150 rpm and an initial pH of 8.0. More interestingly, the optimization was accomplished using two cheap and local fermentation substrates, CF and FF, which could result in a significant reduction in the cost of medium constituents. The maximum alkaline protease production was 9127 U/ml after 72 h of incubation and showed 5-fold increase in protease production over the initial level.

Keywords

***Bacillus mojavensis,* Protease, Chickpea, Faba Bean, Plackett Burman Design**

1. Introduction

Microorganisms are essential sources for enzyme production for industry. Among these enzymes, proteases account for nearly 60% of the total industrial enzyme market [1]. *Bacillus* strains are the main producer for large

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proportion of commercially available proteases.

The use of alkaline proteases has increased remarkably in many industrial processes, including the production of detergents, food processing, in animal feed and for silver regeneration of X-ray films [2]. It is well known that extracellular protease production by microorganisms is greatly influenced by the media components, especially the carbon and nitrogen sources, but also including additional factors such as the temperature, pH, incubation time, agitation and inoculums density [3]. Since approximately 30% - 40% of the production cost in industrial enzyme production is due to medium growth, optimizing the medium composition is especially vital [4].

In general, no defined medium could be carried out for the production of alkaline proteases from different microorganisms; each strain has its specific required conditions for maximum enzyme production [2]. The effect of environmental conditions on the production of extracellular proteolytic enzymes can play an important role in the induction or repression of the enzyme by specific compounds [5]. Protease production is dependent on the availability of both carbon and nitrogen sources within the medium and both of these parameters exert regulatory effects on enzyme synthesis [6]. At present, the cost of enzyme production is very high due to high cost of the substrates and microbiological media used. Therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements, coupled with lowering the production cost, is highly appreciable from a commercial point of view. Therefore, microbial enzymes producing industries are always in search of new and cheaper methods to enhance enzymes production and decrease the market price of the desired enzyme. In this way, efforts have been directed to search for alternatives explore different means to reduce protease production cost through improving the yield and using either cost-free or low-cost feed stocks or agricultural products as substrates for proteases production [7] [8].

Optimization of media compounds by the traditional "one-variable at-a-time" strategy is the most frequently used method in biotechnology [9]. This strategy is extremely time consuming and expensive when a large number of variables need to be considered. Additionally, this method is unable to detect the interactions among different production factors true and then unable to find optimal conditions. Recently, the use of statistical approaches involving Plackett-Burman design has gained momentum in medium optimization [10]-[12]. This method is also useful for understanding interactions among various physico-chemical parameters using a minimal number of experiments. The Plackett-Burman design allows for the screening of the main factors from a large number of variables, and this information can be retained in further optimization.

Bacillus mojavensis A21 has been recently identified as a producer of extracellular bleaching-stable alkaline proteases. Haddar *et al*., 2009 [13] [14] had purified three detergent stable alkaline serine-proteases from the culture supernatant of *B. mojavensis* A21. Otherwise, *B. mojavensis* A21 proteases were used to produce hydrolysates of various proteins [15]-[17]. Haddar *et al*. [18] optimized for *B. mojavensis* A21 culture conditions, by Plackett-Burman and Central Composite Design and the protease production was enhanced by 14 folds on hulled grain of wheat and sardinella peptone based medium.

In view of the promising applicability of the alkaline proteases as a builder for detergents, there is an interest in producing these enzymes in the highest yields with the lowest cost fermentation media. Herein, we report a statistical optimization of culture conditions for the production of proteases by *B. mojavensis* A21, on uncommon fermentation substrates such as faba bean and chickpea flours.

2. Material and Methods

2.1. Microorganism

B. mojavensis A21 producing bleach-stable alkaline proteases was isolated from a marine water sample. It was identified on the basis of 16 S rRNA gene sequencing [10].

2.2. Carbon and Nitrogen Substrates

Seeds of chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lens (*Lens culinaris*), corn (*Zea mays*), pea (*Pisum sativum*) and mil (*Pennisetum glaucum*) were purchased in local market and minced in our laboratory to obtain the corresponding flours: CF, FF, LF, CNF, PF and MF. Sardinella peptone (SP) [19], combined heads and viscera sardinella powder (CHVSP) [20] and hydrolyzed feathers (HF) [21] were prepared in LGEM laboratory.

2.3. Culture Conditions

B. mojavensis A21 was maintained on Luria-Bertani (LB) medium [22]. The proteolytic enzymes were initially

produced on a basal medium composed of g/l: Yeast extract (YE) 1, NaCl 2, K_2HPO_4 1, KH_2PO_4 0.3, $MgSO_4$ 1, and supplemented with a carbon source at 30 g/l. The cultures were conducted at pH 8, 30˚C and 200 rpm for 48 hours in 250 ml Erlenmeyer flasks with a working volume of 25 ml. The culture medium was centrifuged at 10,000 g for 15 min at 4˚C and the cell free supernatant was used for estimation of proteolytic activity.

2.4. Protease Assay

Protease activity was measured according to the method of Kembhavi *et al*. (1993) [23] using casein as a substrate. A sample of 0.5 ml suitably diluted enzyme, was mixed with 0.5 ml of 100 mM glycine-NaOH, pH 10.0 containing casein at 1% (w/v) and incubated for 15 min at 60˚C. The reaction was stopped with the addition of 0.5 ml of trichloroacetic acid (20%; w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 g for 15 min to remove the precipitate. The absorbance of the soluble fraction was measured at 280 nm. A standard curve was generated using solutions of 0 - 50 mg/l of tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions used.

2.5. Experimental Designs

A Plackett-Burman design is used for rapid screening multifactor to find the most significant independent factors [5] [24] [25]. The independent variables of the protease production were Chikpea flour (CF), Faba bean flour (FF), K_2HPO_4, KH_2PO_4, $CaCl_2$, NaCl, $MgSO_4$, incubation temperature, speed of agitation, incubation time and inoculums size (IS). Then the eleven factors were investigated using the Plackett-Burman design with a first-order polynomial equation. Each factor was tested at low (−1) and high (+1) levels (**Table 1**). Eleven variables were screened in 16 experimental runs in which 4 runs were repeated at the central level (0) (**Table 2**). The fitted first-order model is: $Y = \beta_0 + \sum \beta_i X_i$; Y is the predicted response, β_0 and β_i are constant coefficients, and X_i is the coded independent factors.

Box-Behnken design of RSM (Response Surface Methodology) [25] [26] was employed to optimize levels of the five selected factors (temperature, CF, FF, incubation time and inoculums size) for enhancing the protease production. The five independent factors were investigated at three different levels (−1, 0, +1) (**Table 3**) and the experimental design used for the study is shown in **Table 4**.

The protease production was fitted using a second-order polynomial equation and a multiple regression of the data was carried out for obtaining an empirical model related to the factors. The general form of the second-order polynomial equation is:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear

Table 1. Levels of the factors tested in Plackett-Burman design.

Variables	Units	Symbol code	Lower (−1)	Higher (+1)
CF	g/l	X_1	10	40
FF	g/l	X_2	10	30
$CaCl_2$	g/l	X_3	0.5	2
NaCl	g/l	X_4	0.5	2
KH_2PO_4	g/l	X_5	0.1	1
K_2HPO_4	g/l	X_6	0.1	1
$MgSO_4$	g/l	X_7	0.1	1
Temperature	˚C	X_8	30	37
Speed of agitation	rpm	X_9	150	250
Time	H	X_{10}	24	72
IS	%	X_{11}	2	10

CF: chickpea flour; FF: faba bean flour and IS: inoculums size.

Table 2. Plackett-Burman design for screening of significant factors affecting protease production levels.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Proteolytic activity (U/ml)
1	1	1	−1	1	1	1	−1	−1	−1	1	−1	9127
2	−1	1	1	−1	1	1	1	−1	−1	−1	1	463
3	1	−1	1	1	−1	1	1	1	−1	−1	−1	663
4	−1	1	−1	1	1	−1	1	1	1	−1	−1	1227
5	−1	−1	1	−1	1	1	−1	1	1	1	−1	236
6	−1	−1	−1	1	−1	1	1	−1	1	1	1	1190
7	1	−1	−1	−1	1	−1	1	1	−1	1	1	1163
8	1	1	−1	−1	−1	1	−1	1	1	−1	1	1581
9	1	1	1	−1	−1	−1	1	−1	1	1	−1	8481
10	−1	1	1	1	−1	−1	−1	1	−1	1	1	2045
11	1	−1	1	1	1	−1	−1	−1	1	−1	1	2281
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	636
13	0	0	0	0	0	0	0	0	0	0	0	2581
14	0	0	0	0	0	0	0	0	0	0	0	2490
15	0	0	0	0	0	0	0	0	0	0	0	2527
16	0	0	0	0	0	0	0	0	0	0	0	2493

Variables X_1 to X_{11} are described in **Table 1**.

Table 3. Levels of the factors tested in Box-Behnken design.

Factor	Units	Levels		
		−1	0	1
CF	g/l	10	25	40
FF	g/l	10	20	30
Time	H	24	48	72
Temperature	˚C	30	33,5	37
IS	%	2	6	10

coefficient, β_{ii} is the quadratic coefficient and β_{ii} is the interaction coefficient.

Design-expert, version 7.0 (STAT-EASE Inc., Minneapolis, USA) was used for the experimental designs and statistical analysis of the experimental data. The analysis of variance (ANOVA) was used to estimate the statistical parameters.

2.6. Chemical Composition of CF and FF

Dry weights of chickpea and faba bean flours were determined after heating samples at 105˚C to constant weight, and then ash content was determined after heating dried samples at 600˚C for 2 h. Total nitrogen was determined using the Kjeldahl method and then protein content was estimated. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with diethyl ether. Starch and cellulose were estimated as described by Ewers [27] and Gautier [28] respectively.

Table 4. The Box-Behnken design of RSM for optimization of the protease production by *B. mojavensis* A21.

Run	CF	FF	Time	Temperature	IS	Proteolytic activity (U/ml)	
						Experimental	Predicted
1	10	10	48	33.5	6	672	624
2	40	10	48	33.5	6	2372	2071
3	10	30	48	33.5	6	1218	1451
4	40	30	48	33.5	6	3745	2898
5	25	20	24	30	6	1290	1608
6	25	20	72	30	6	2736	2671
7	25	20	24	37	6	1554	1601
8	25	20	72	37	6	1500	1164
9	25	10	48	33.5	2	1390	1348
10	25	30	48	33.5	2	1936	2174
11	25	10	48	33.5	10	1300	1348
12	25	30	48	33.5	10	2290	2174
13	10	20	24	33.5	6	1854	1347
14	40	20	24	33.5	6	1654	1862
15	10	20	72	33.5	6	1090	728
16	40	20	72	33.5	6	2754	3107
17	25	20	48	30	2	1909	2139
18	25	20	48	37	2	1354	1383
19	25	20	48	30	10	2081	2139
20	25	20	48	37	10	918	1383
21	25	10	24	33.5	6	954	1191
22	25	30	24	33.5	6	1654	2018
23	25	10	72	33.5	6	1272	1504
24	25	30	72	33.5	6	2500	2331
25	10	20	48	30	6	1190	1416
26	40	20	48	30	6	2500	2863
27	10	20	48	37	6	900	659
28	40	20	48	37	6	2509	2106
29	25	20	24	33.5	2	2290	1605
30	25	20	72	33.5	2	2018	1917
31	25	20	24	33.5	10	1845	1605
32	25	20	72	33.5	10	1727	1917
33	10	20	48	33.5	2	1036	1038
34	40	20	48	33.5	2	3127	2484
35	10	20	48	33.5	10	1263	1038
36	40	20	48	33.5	10	2136	2484
37	25	10	48	30	6	2054	1726
38	25	30	48	30	6	2918	2553
39	25	10	48	37	6	763	970
40	25	30	48	37	6	1127	1796
41	25	20	48	33.5	6	1681	1761
42	25	20	48	33.5	6	2000	1761
43	25	20	48	33.5	6	1563	1761
44	25	20	48	33.5	6	1254	1761
45	25	20	48	33.5	6	1672	1761
46	25	20	48	33.5	6	1436	1761

3. Results and Discussion

3.1. Effect of Carbon Sources on Protease Production

B. mojavensis A21 strain was cultivated on initial basal medium containing YE at 1 g/l as nitrogen source and different complex sources of carbon (30 g/l) such as chickpea, faba bean, lens, corn, pea and mil. As shown in **Figure 1**, *B. mojavensis* A21 was found to produce proteolytic enzymes in the presence of complex organic sources. Among these, chickpea flour (CF) and faba bean flour (FF) gave the highest proteolytic activities of 2995 U/ml and 2350 U/ml respectively. Various concentrations of CF and FF were then tested for the proteases production by *B. mojavensis* A21. It was shown that the proteolytic activity was highly stimulated when increasing the concentrations of CF. This production reached 4336 U/ml on CF at 60 g/l and then it was stabilized (**Figure 2**).

Our results are in concordance with other reports regarding proteases production, in which it has been observed that complex carbon sources such hulled grain of wheat [12], soybean meal [29] or fish powder [30], were efficiently used for protease production by microorganisms. Such products constitute better substrates for enzyme production than simple sugars like glucose which can induce catabolic repression [31].

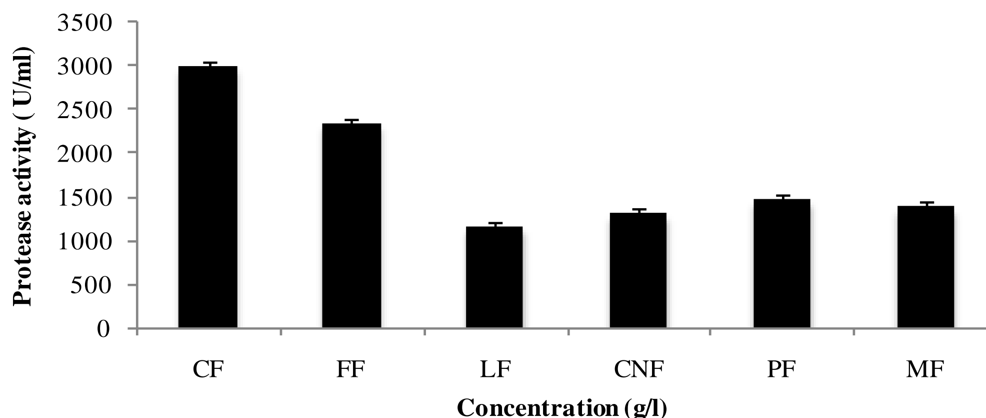

Figure 1. Effect of different carbon sources on protease production by *B. mojavensis* A21 at 37˚C in the basal medium (pH 8.0) supplemented with different carbon sources at 30 g/l. Flours were obtained from chickpea: CF, faba bean: FF, lens: LF, corn: CNF, pea: PF and mil: MF.

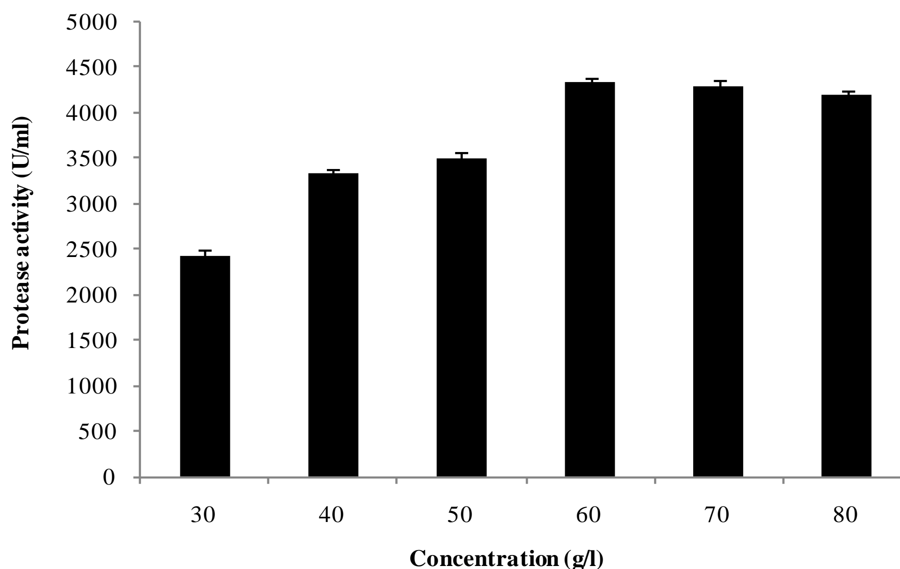

Figure 2. Effect of increasing concentration of CF on protease production by *B. mojavensis* A21 in the basal medium.

3.2. Effect of Nitrogen Sources

In a second experiment, protease production by *B. mojavensis* A21 was checked in the basal medium containing CF at 30 g/l and supplemented with 1 g/1 of various nitrogen sources YE: yeast extract, SP: sardinella peptone, CHVSP: combined heads and viscera sardinella powder, HF: hydrolyzed feathers. As shown in **Figure 3**, the bacteria exhibited high levels of enzyme activity of 3000 U/ml and 2850 U/ml when the medium was supplemented with YE or SP respectively. The supplementation of medium with enzymatically hydrolyzed proteins CHVSP or HF had a negative influence on the enzyme production which drops to 1081 and 1000 U/ml respectively.

In order to substitute YE by a low cost and available nitrogen source, FF was assayed at different concentrations from 10 to 40 g/l in basal medium containing 40 g/l of CF as carbon source. Protease production was enhanced with the increase of FF concentration up to 30 g/l (**Figure 4**), reached 4645 U/ml, and then decreased beyond.

In this study, two kinds of legume seeds were shown to be excellent carbon and nitrogen sources for *B. mojavensis* A21 proteases production which was maximized in the presence of CF. This can be explained by the fact that the two respective flours contain high amounts of proteins.

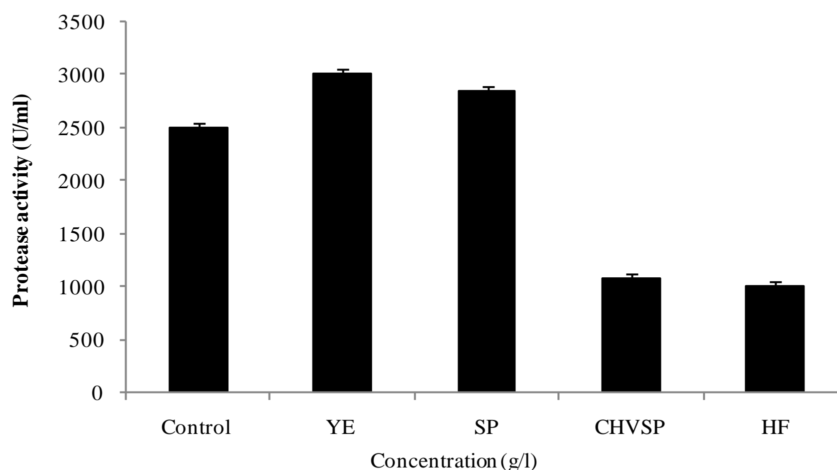

Figure 3. Effect of different nitrogen sources on protease production by *B. mojavensis* A21 Cultures were conducted at 37˚C (pH 8.0) in the basal medium containing CF at 30 g/l and supplemented (or not) with one of the following nitrogen sources (30 g/l): YE: yeast extract, SP: sardinella peptone, CHVSP: combined heads and viscera sardinella powder, HF: hydrolyzed feathers.

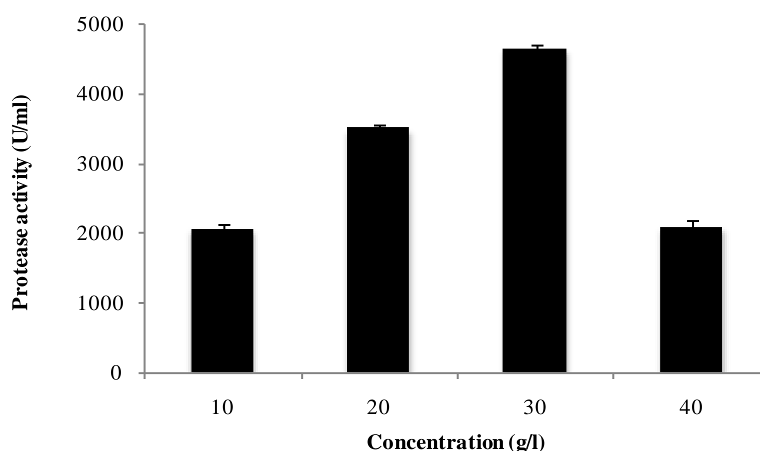

Figure 4. Protease production by *B. mojavensis* A21 on medium containing CF at 40 g/l supplemented with various concentrations of FF.

3.3. Screening of Significant Variables Using Plackett-Burman Design

The Plackett-Burman design is a powerful method for screening significant factors. Sixteen runs were carried out to analyze the effect of 11 variables on protease production and the results are demonstrated in **Table 2**. The t-test was used to identify the effect of every factor on protease production. **Figure 5** shows that CF, FF, temperature, incubation time and IS are the significant factors ($p < 0.05$). According to the model, CF, FF, NaCl, Speed agitation and incubation time exerted a positive effect. However, $CaCl_2$, KH_2PO_4, K_2HPO_4, $MgSO_4$, temperature and IS exerted negative effects on the protease production. CF, FF, temperature, incubation time and IS were selected for further optimization to obtain a maximum response. $CaCl_2$, KH_2PO_4, K_2HPO_4 and $MgSO_4$ have been fixed to the low level corresponding to 0.5 g/l, 0.1 g/l, 0.1 g/l and 0.1 g/l, respectively. The NaCl and speed agitation have been fixed to the levels corresponding to 2 g/l and 250 rpm, respectively.

3.4. Optimization of Significant Variables Using Variance (ANOVA) Analysis

A response surface design is further applied when the optimal region for running the process has been identified. Based on the Plackett-Burman design, RSM using Box-Behnken design was applied to determine the optimal levels of the five selected variables (CF, FF, temperature, incubation time and IS) which significantly influenced the protease production. The respective low and high levels with the coded levels for the five variables are defined in **Table 3**. A total of 46 runs with different combination of CF, FF, temperature, incubation time and IS were performed (**Table 4**). The experimental resultants were analyzed by standard ANOVA (**Table 5**) and the Box-Behnken design was fitted with the second-order polynomial equation (in coded factor):

$$Y = 1761(\pm 52.93) + 723.375(\pm 89.75)\text{ CF} + 413.1875(\pm 89.75)\text{ FF} + 156.375(\pm 89.75)\text{ Time} - 378.3125(\pm 89.75)\text{ Temperature} + 466(\pm 179.50)\text{ CF} \times \text{Time} - 375(\pm 179.50)\text{ Time} \times \text{Temperature}$$

where Y the protease production by *B. mojavensis* A21.

The statistical significance of the model equation was evaluated by the F-test for ANOVA. The model F-value of 19.68 implies the model is significant. There was only a 0.01% chance that the model F-value could occur due to noise. The lack of fit F-value of 2.16 implies that was not significant relative to the pure error and that was a 19.83 % chance that the lack of fit F-value could occur due to noise. The adequate precision measures the

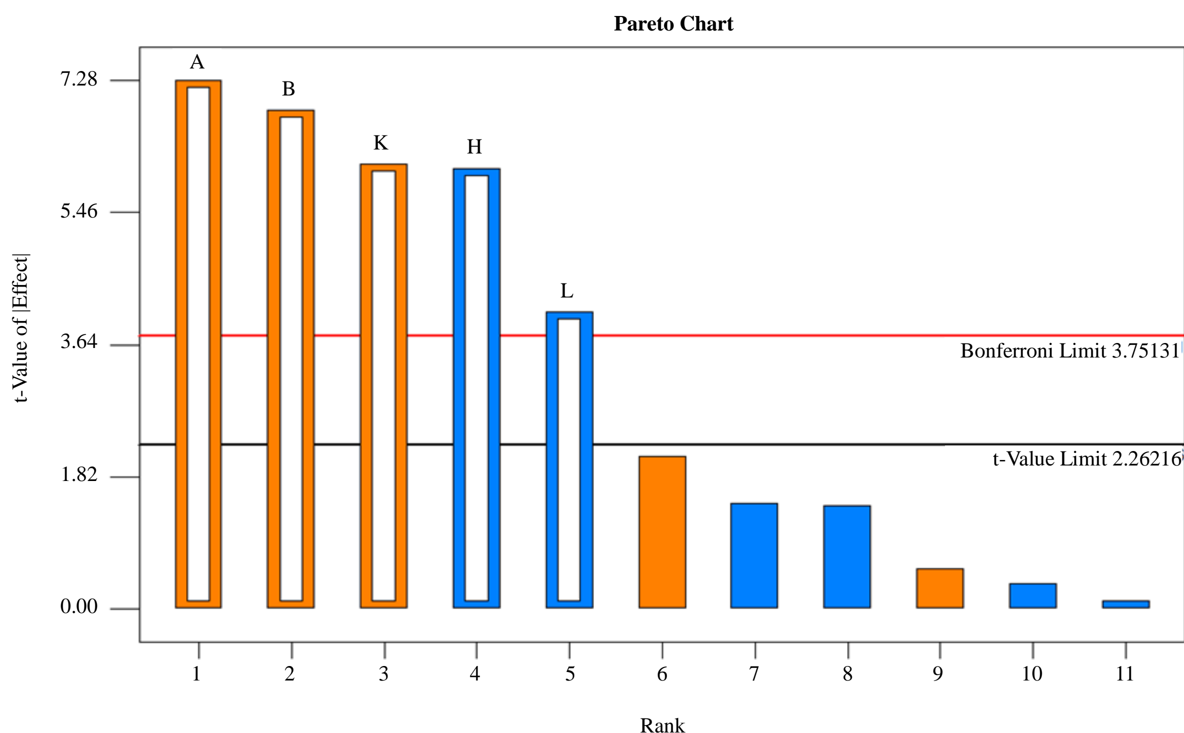

Figure 5. Identification of significant variables for proteases production by *B. mojavensis* A21 using Plackett-Burman design (Pareto chart); where A is CF, B is FF, H is temperature, K is time and L is inoculums size.

Table 5. Analysis of variance (ANOVA) for response surface reduced 2FI Model.

Source	Sum of squares	Df	Mean square	F-value	p-value
Model	**15,216,225**	**6**	**2536037.4**	**19.68**	**<0.0001***
CF	8,372,342	1	8372342.3	64.96	<0.0001*
FF	2,731,583	1	2731582.6	21.20	<0.0001*
Time	391,250	1	391250.3	3.04	0.0893
Temperature	2,289,926	1	2289925.6	17.77	0.0001*
CF × Time	868,624	1	868624.0	6.74	0.0132*
Time × Temperature	562,500	1	562500.0	4.36	0.0433*
Residual	**5,026,193**	**39**	**128876.8**		
Lack of fit	4,706,473	34	138425.7	2.16	0.1983
Pure error	319,720	5	63944.0		
Cor Total	**20,242,418**	**45**			

Standard Deviation = 358.99; R^2 = 0.752, adjust R^2 = 0.714; *Statistically significant at 95% of confidence level.

signal to noise ratio. A ratio greater than 4 is desirable. Adequate precision which was calculated to be 17.73 indicates an adequate signal. This model can be used to navigate the design space.

According to the model, we can notice that the increasing of the temperature causes to a decrease in the protease production. **Table 5** shows that only CF, FF, temperature, incubation time, the interaction CF × Time and the interaction Time × Temperature are significant however, IS is not significant. The interaction CF × Time exerted a positive effect on the protease production, whereas the interaction Time × Temperature exerted a negative effect on the protease production. Among the five significant variables (CF, FF, incubation time, temperature and IS), CF concentration was found to have the greatest effect on the production of alkaline proteases. The optimal values of the five variables predicted by the model are CF = 38.08 g/l, FF = 27.49 g/l, time = 67.68 h, temperature = 30.26˚C and IS = 3.37. The maximum predicted protease production was 3798.67 ± 435.63 U/ml.

In a first attempt to optimize *B. mojavensis* A21 proteases production by statistical design on wheat bran flour and sardinella peptone, Haddar [13] obtained 1783 U/ml. Herein, we showed that protease production by *B. mojavensis* A21 was enhanced by 5 folds when cultivated on the optimized medium based on chickpea and faba bean flours used as carbon and nitrogen sources. Similarly, several works related the optimization of proteases production by statistical design for various *Bacillus* strains [30] [32]-[35].

Using cost-effective media formulation and optimizing this media to determine its minimum requirements for maximum enzyme production is extremely important in industrial-scale protease production for economic reasons. Therefore, using common and local low cost substrates such as chickpea and faba bean, will serve as a potential example for the applications in industrial microbial fermentations. The simple expedient of replacing YE in the medium with CF or FF could significantly lower the production cost.

Maximum proteases production of 9127 U/ml was achieved at the optimized culture conditions: 40 g/l CF, 30 g/l FF, NaCl 2 g/l; KH_2PO_4 1 g/l; K_2HPO_4 1 g/l; $CaCl_2$, 0.5 g/l; $MgSO_4$ 0.1 g/l and IS 2% (v/v). In addition other factors were taken at suitable levels, as shown in the Plackett-Burman design matrix: a temperature of 30˚C, a speed of agitation at 150 rpm, incubation time of 72 h and an initial pH of 8.0.

3.5. Time Courses of Protease Production and Cell Growth

The time courses of protease activity and the growth of *B. mojavensis* A21 under the optimized conditions are shown in **Figure 6**. The kinetics showed that the secretion of the proteases was associated with the bacterial growth. Protease activity increased during the exponential growth phase and reached a proteolytic activity of 9127 U/ml at the end of the stationary phase, after 72 h of growth. The kinetics of growth and protease production showed that the majority of alkaline proteases (7500 U/ml) were secreted by *B. mojavensis* A21 at the end of the exponential growth phase of the bacteria.

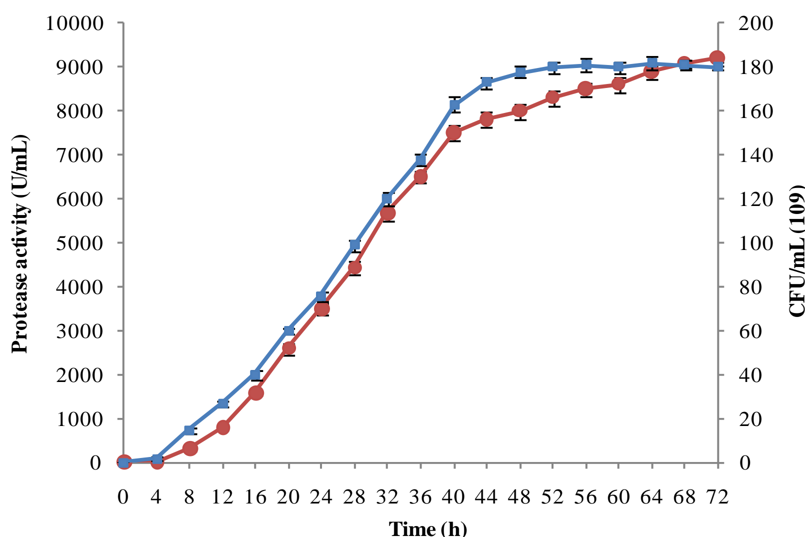

Figure 6. Kinetics of growth and alkaline proteases production by *B. mojavensis* A21 in the optimized medium consisting of (g/l): CF 40; FF 30, NaCl 2.0; KH_2PO_4 1.0; K_2HPO_4 1; $CaCl_2$ 0.1; $MgSO_4$ 0.1; pH 8.0 and IS 2%. (●) Protease activity (U/ml) and (▬) biomass (CFU/ml).

Table 6. Chemical composition of CF (chickpea flour) and FF(faba bean flour).

Components %	FF	CF
Proteins	29.08 ± 1.55	23.64 ± 1.07
Fat	2.18 ± 0.26	6.48 ± 0.42
Humidity	13.04 ± 0.77	15.35 ± 0.80
Starch	42.24 ± 1.28	46.91 ± 0.64
Cellulose	9.61 ± 0.84	3.90 ± 1.27
Ash	3.85 ± 0.21	3.72 ± 0.50

3.6. Chemical Compositions of CF and FF

The chemical compositions of CF and FF were determined (**Table 6**). Both FF and CF exhibited high content of proteins, 29.08% and 23.64%, and starch 42.24% and 46.91% respectively. While FF is richer in cellulose and had a lower fat content than CF. Though, proteins and starch represent both more than 70% of raw material for the two pulses.

The two pulses contained important quantities of proteins and starch which are higher than in hulled grain of wheat (HGW) 8% - 12% and 50% - 60% respectively [12]. Our results and those of Haddar *et al*. [13] clearly indicated that proteins and starch are useful for protease production. Herein the proteases production by *B. mojavensis* A21 strain, was amplified by the use of highly containing proteins substrates such FF and CF. This enhancement could be explained by the higher content of proteins compared to the previously used raw material: HGW. More proteins probably stimulate more proteases synthesis.

Thus, the use of CF and FF as carbon and nitrogen sources may result in a more cost-effective process, and so the production of detergent proteases by the A21 strain will be effectively economic.

4. Conclusion

Due to the increasing economic relevance of alkaline proteases, this study was conducted in an attempt to optimize a variety of fermentation parameters, including medium compositions and culture conditions, for maximal alkaline protease production. Eleven variables were tested using the Plackett-Burman design, and five variables

(CF, FF, incubation time, temperature and IS) were selected as the most influential factors on enzyme production. The maximum alkaline protease production was amplified by 5 folds over the initial level and reached 9127 U/ml after 72 h of incubation. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents and would thus offer advantages for large-scale fermentations.

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