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A novel proteolytic fungus, statistical screening of medium components for amino acids production

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Abstract: Soybean as a cheap and aliquot agricultural by-products have the ability in applying the entire SSF process. Evaluation of the effective conditions for amino acid production, the statistical experimental design of Pl ackett–Burman was applied for the improvement of protease yield by Aspergillus flavus using soybean residuals as raw materials. Plackett–Burman design had been applied to value the effects of six variables: independent variable soybean residuals as a substrate, spore suspension, the incubation period, pH, MgSO4.7H2O, (NH4)2SO4 at high concentration for all factors as 13 day, 6 mL/g and 2.2 mL/g respectively. The key factors influencing protease production and production of free amino acids were identified as incubation period, the concentration of KH2PO4, and concentration of (NH4)2SO4. The statistical experimental design was proven as an effective pathway to obtain optimum parameters for amino acids production

keywords: Aspergillus flavus, Plackett–Burman design, protease, soybean, plant residuals

1.Introduction

Enzymes are great macro-molecules composed of polymer chains from amino acids connected by each other through amide bonds. They are recognized as biocatalysts that perform an abundance of chemical reactions **[1, 2]**. Proteases are one of the significant substantial series of industrial enzymes that had been studied extensively through recent years **[3]**. Proteases (EC 3.4.21.24) are group of enzymes that secrete in all living organisms such as viruses, animals, and also humans as well as occur ubiquitously in a wide variety of microorganisms **[4, 5]**. Using cheap sources from nitrogen or carbon as marine products waste, rice bran, wheat bran, gelatin, soybean, ocean waste, agro-based products are important as they could respectably minimize the cost of the valuable products like enzymes and proteins **[6]**.

Solid-state fermentation (SSF) is a process mentioned to microbial fermentation, which carried out in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted. In recent years, SSF is an economically applicable, practically acceptable technology widely for bioconversion and biodegradation processes. Based on sustainable SSF bioprocess technology will emerge multidisciplinary scales have handling application for bioethanol production, enzyme production, chemicals manufacturing and pharmaceuticals **[7,8].**

Optimization of media components by classical methods “one-factor-at-a-time (OFAT)” experiments, only changing in one variable or factor while regarding other variables were constant with time. Classical methods involve the change of a single variable but such optimization strategies have some disadvantages, such as time-consuming, the requirement of more experimental data sets, and missing the interactions among variables **[9]**. It may lead to incorrect results and inaccurate conclusions. These traditional

methods were comprise a relatively great numbers of experiments, which lead to laborious and time-consuming, especially in multinomial factors cases **[10]**.

On the contrary, Plackett–Burman design "PBD" is considered to be more influential and potent in screening the major factors of multivariable system to adaptable fermentation of multi-conditions and they are an efficient experimental strategy to determine the optimal conditions for the multivariable system as will be error-proof also time-saving in response design **[11]**. This work aims to study the most active proteolytic fungal isolates associated with one of the most abundant agrowaste in the world. Furthermore, determining which fungal species have the ability to usage of them for biodegradation of nonconventional proteinaceous wastes as soybean residues.

2. Materials and Methods

2.1. Preparation of Soya bean sample for isolation of microbial strains:

Soybean hulls was obtained from Tag Elezz Research Station, Dakahlia Province, Egypt. All substrates had been kept in an airtight container then stored in cold room for future use. The air-dried samples were cut into 0.5-1 cm. The fragments of residuals were transferred to plates with Potato Dextrose Agar medium (PDA) to isolate fungi only. After obtaining pure isolates of fungi maintenance of pure fungal PDA slants in the refrigerator for identification and further studies.

2.2. Identification of fungal isolates:

Fungal genera and primary identification of species were carried out through their microscopical, morphological and cultural properties, look like (appearance of the colonies, color, texture and colony diameter) **[12-16]**.

2.3. Screening of isolates for protease production on broth medium

Fungal isolates were screened for the production of protease on the broth medium of **[17]** with the following composition per (g/L): K_2HPO_4 (1.0) g, $MgSO_4.7H_2O$ (0.5) g, Casin "skim milk" (20) g. Using 20 g L^{-1} casein as a sole source of carbon and nitrogen. pH of medium was adjusted at 6.0 to 6.5 with 1 N, NaOH before autoclaving. Added three discs "1cm for each disc" of previous prepared PDA inoculums of each isolate to inoculate Erlenmeyer flasks"250 mL" containing 20 mL of sterilized medium. Flasks had been incubated with shaking (120 rpm) at 28 °C for 7 days; then flasks were centrifuged for ten minutes at 4000 rpm. Culture filtrate was tested for protease.

Protease Enzyme assays

The protease activity of the crude proteases was determined by using the modified Rick's method **[18]**. In cleaned test tubes (0.5 ml of crude enzyme) were added to (2.5 ml) of 0.4% casein in (0.2 M) Tris HCl (pH 9.0) buffer and incubated at 37° for 30 min as a substrate solution. Then added about two ml 10% TCA (Trichloroacetic acid), and leave for standing at room temperature for 30 min. The solution was centrifuged at 120 rpm for 5 min and filtered. Take 1 ml of filtrate with 5 ml of water was added, the optical density (O.D.) at 300 nm was determined by using a UV spectrophotometer. The blank tube was prepared by adding 10% TCA to each crude enzyme before the addition of casein substrate buffer. One unit of protease activity could defined as the amount of enzyme-producing 1 mL tyrosine per min under assay conditions.

2.5 Determination of free amino acid in broth media

In cleaned test tubes, one ml of TCA was added to one ml of supernatant while test tubes were centrifuged at 5000 rpm for five min (measure absorbance (A) at 300 nm before protease enzyme estimation) using Tyrosine standard curve.

2.6. Fermentation medium and culture conditions of Solid-State Fermentation "SSF" media

2.6.1 Preparation of fungal spore inocula and media

Five millimeter of sterile distilled water was added to 7 days sporulated old PDA slant cultures. Spores were scraped by usage of an inoculating needle in aseptic conditions to obtain homogeneous spore suspension. Soybean agricultural residuals were used separately as a sole source of protein and carbon at the same time. Fermentation media containing one-gram residuals which would be

hydrated with distilled water to desired moisture (w/v) then added 1.5 mL of salt solution to each flask. A **[19]** salt solution media composed of per (g/L): (KH_2PO_4 4.0 g), ($MgSO_4.7H_2O$ 1.0 g), and ($(NH_4)_2SO_4$ 1.6 g). The media will be modified by removing ($(NH_4)_2SO_4$) to obligate fungi for using soybean residuals as a sole source of carbon and nitrogen. All components were added separately to the Erlenmeyer flask and autoclaved at 121^oC for 20 minutes. After autoclaving, 1 mL of spore suspension was inoculated to each flask. The initial moisture content was adjusted to approximately about 65 % and cultivated at 30 ºC for 10 to 14 days. The crude enzyme solution was obtained by adding 10 mL of Tween 80 solution to each flask. Contents were mixed by shaking for 30 min on a rotatory shaker at 140 rpm. Extract solution was filtered through Whatman No.1 then filtrate was used as the crude enzyme.

2.6.2 Estimation of protease enzyme and total amino acids

The filtrate was used for the determination of the protease enzyme, free amino acids as previous methods and final pH. Enzymatic activity for SSF was calculated as follows: Unit/gm of soybean waste.

2.7 Screening of medium components for AA production using Placket-Burmann Design (PBD)

Major processing factors impacted in production of amino acids over SSF. PBD strategy carried out to optimize six independent variables with the optimal conditions for amino acid production. In this research, six factors were studied. Variables were spore suspension (S-A) ml/g, incubation period (IP-B) day, pH range (pH-C), KH_2PO_4 (K-D) mg/g Waste, $MgSO_4.7H_2O$ (M-E) mg/g Waste, $(NH_4)_2SO_4$ (N-F) mg/g Waste. According to PBD, each parameter was prepared using two levels: −1 refer to low level and +1 for high level. Table (1) mentioned to levels of each factor in experimental design. Table (2) represented the design matrix. Statistical analysis was performed to obtain the most precise and comprehensive experimental design by using the software package Minitab (version 19.0)

Table1. Levels of independent variables applied for Plackett-Burmann design optimization experiment

Independent variable (g)	Spore suspension S (A) mL /g waste)	Incubation period IB (B) (day)	pH range (C)	KH_2PO_4 (D) (mg/g waste)	$MgSO_4.7H_2O$ (E) (mg/g waste)	$(NH_4)_2SO_4$ N (F) (mg/g waste)
Low level	0.5	7	5.0	2.0	0.5	1.0
Center level	1.0	10	7.0	4.0	1.0	1.6
High level	1.5	13	9.0	6.0	1.5	2.2

Results

Ten fungal species were isolated from soybean residuals and identified based on their morphology.

3.1. Isolation and protease activity of fungi on broth media

With regard to trials isolation, ten fungi comprise four genera were isolated from soybean residuals. They were coded from one to ten. All these isolates were screened for protease activity on broth media contain casein as a sole source of protein and data were recorded as shown in table (4) also measuring of free amino acids and final pH of supernatant

Isolates have protease activity values that were taken in the considerations with the coded number (3, 6, 10 and 9); also, these isolates have raised the ratio of free amino acids as isolate no. 3 and 8 respectively. All isolates appeared to increase the pH except isolate no.2 and 10.

3.2. Quantification of protease activity and production of amino acids on SSF media

The results in Table (5) indicated that the isolated fungal species showed variations in protease activity also in the production of amino acids. The most active isolate for protease activity was *Aspergillus* sp. MW9, has the highest value in enzyme production followed by *Aspergillus* sp.MW3 and *Penicillium* sp.MW6**.** On the other hand, *Trichoderma* sp. MW10 showed the weakest protease activities 0.745U mL^{-1} gm soybean residuals, respectively. For the production of free amino acids and value of pH *Aspergillus* sp.MW9 isolate followed by *Trichoderma* sp. MW10

Table:2 Plackett–Burman experimental design matrix for evaluated coded and uncoded values of six factors influencing amino acid production

Tested Independent variables													Free A.As	predicted
Variables	Spore suspension (A) mL/g		Incubation period (B) per day		pH values (C)		KH_2PO_4 (D) mL/g		$MgSO_4.7H_2O$ (E) mL/g		$(NH4)_2SO_4$ (F) mL/g			
Runs	Coded value	Actual value	Coded value	Actual value	Codd value	Actul value	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value		
1	1	2	-1	7	1	9	-1	2	-1	1	-1	1	36.48	36.94
2	1	2	1	13	-1	5	1	6	-1	1	-1	1	43.73	43.25
3	-1	1	1	13	1	9	-1	2	1	2	-1	1	45.11	44.37
4	1	2	-1	7	1	9	1	6	-1	1	1	2.2	39.53	40.07
5	1	2	1	13	-1	5	1	6	1	2	-1	1	36.02	35.26
6	1	2	1	13	1	9	-1	2	1	2	1	2.2	38.86	39.84
7	-1	1	1	13	1	9	1	6	-1	1	1	2.2	44.56	44.38
8	-1	1	-1	7	1	9	1	6	1	2	-1	1	45.59	45.77
9	-1	1	-1	7	-1	5	1	6	1	2	1	2.2	36.02	35.34
10	1	2	-1	7	-1	5	-1	2	1	2	1	2.2	42.82	43.06

Table 3. Protease activity of fungal isolates, free amino acids, and final pH in broth media contain casein

Fungus	Protease(Unit/mL)	Total AA (Unit/mL)	pH
Aspergillus sp. MW1	0.29±0.04	0.31±0.0345	8.2
Fusarium sp. MW2	0.07±0.037	0.14±0.085	5.5
Aspergillus sp. MW3	2.41±0.51	0.57±0.0475	6.5
Penicilliums sp. MW4	0.14±0.02	0.14±0.075	8.0
Bipolaris sp. MW5	0.09±0.01	0.22±0.0275	7.8
Penicillium sp. MW6	1.81±0.29	0.17±0.0485	7.0
Aspergillus sp. MW7	0.19±0.05	0.11±0.184	6.8
Aspergillus sp. MW8	0.23±0.039	1.46±0.363	7.2
Aspergillus sp. MW9	1.43±0.03	0.22±0.151	7.2
Trichoderma sp. MW10	1.73±0.15	0.28±0.084	4.6

Table 4. Protease activity of fungal isolates, free amino acids, and final pH in broth media contain casein

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Aspergillus sp. MW1	0.29±0.04	0.31±0.0345	8.2
Fusarium sp. MW2	0.07±0.037	0.14±0.085	5.5
Aspergillus sp. MW3	2.41±0.51	0.57±0.0475	6.5
Penicilliums sp. MW4	0.14±0.02	0.14±0.075	8.0
Bipolaris sp. MW5	0.09±0.01	0.22±0.0275	7.8
Penicillium sp. MW6	1.81±0.29	0.17±0.0485	7.0
Aspergillus sp. MW7	0.19±0.05	0.11±0.184	6.8
Aspergillus sp. MW8	0.23±0.039	1.46±0.363	7.2
Aspergillus sp. MW9	1.43±0.03	0.22±0.151	7.2
Trichoderma sp. MW10	1.73±0.15	0.28±0.084	4.6

Table 5. Protease activity of fungal isolates, free amino acids and final pH on Solid-State Fermentation (SSF)

Fungus	Protease (Unit/gm)	Free AA (Unit/gm)	pH
Aspergillus sp. MW3	7.00±1.22	6.27±0.77	5.9
Penicillium sp.MW6	4.41±0.89	7.03±1.45	5.9
Aspergillus sp.MW9	12.91±1.5	7.52±1.76	6.2
Trichoderma sp. MW10	0.745±0.18	6.97±1.66	6.5

3.3 Statistical screening of medium components using PBD

PBD applied for survey the most important parameter of tested variables during SSF medium on production of free amino acid of coded sample *Aspergillus* sp.MW9 which have the highest level of free amino acid of SSF matrix and the maximum value of protease activity. Depending on Plackett-Burman matrix, results in Table (2) mentioned to moderate variations in protease production, which ranged from 36.02 U g^{-1} (runs number 5 and 9) to maximum harvesting in run no 8, which listed 45.59 U g^{-1} with a 9.57-fold increase.

PBD statistical analysis for obtained results illustrated in Pareto chart **Fig (1**) and Table (5). It is clear that KH_2PO_4 (6.0 mg/g), incubation period (13 day) and ((NH_4) SO_4 1.0 mg/g) were the respectable variables on amino acid production. Other variables didn't record any obvious impact on responded variables. Furthermore, pH degree, the volume of spore suspension, and $MgSO_4.7H_2O$ indicated to insignificant effect on the production of amino acid which as shown in **Fig (1)** by the standardized impact on the Pareto chart and also in factoria regression of **Table (5)**.

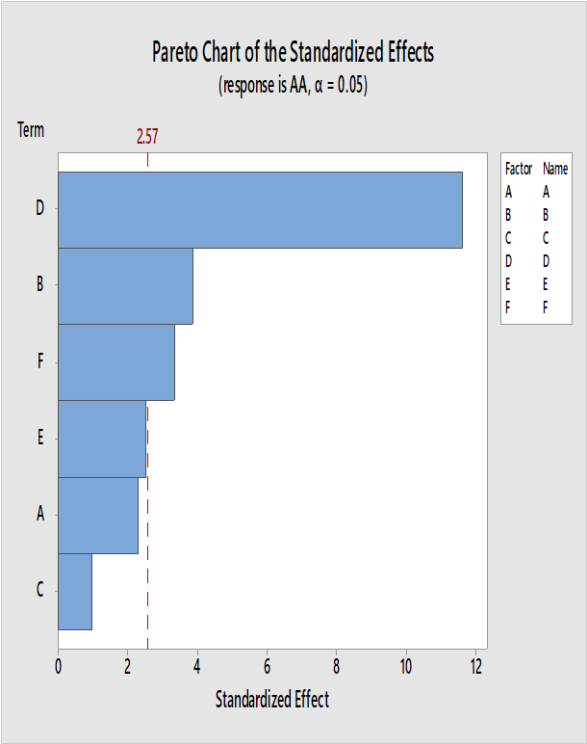

Fig. 1: Pareto chart of standardized effectiveness for the tested parameters on Amino Acid production

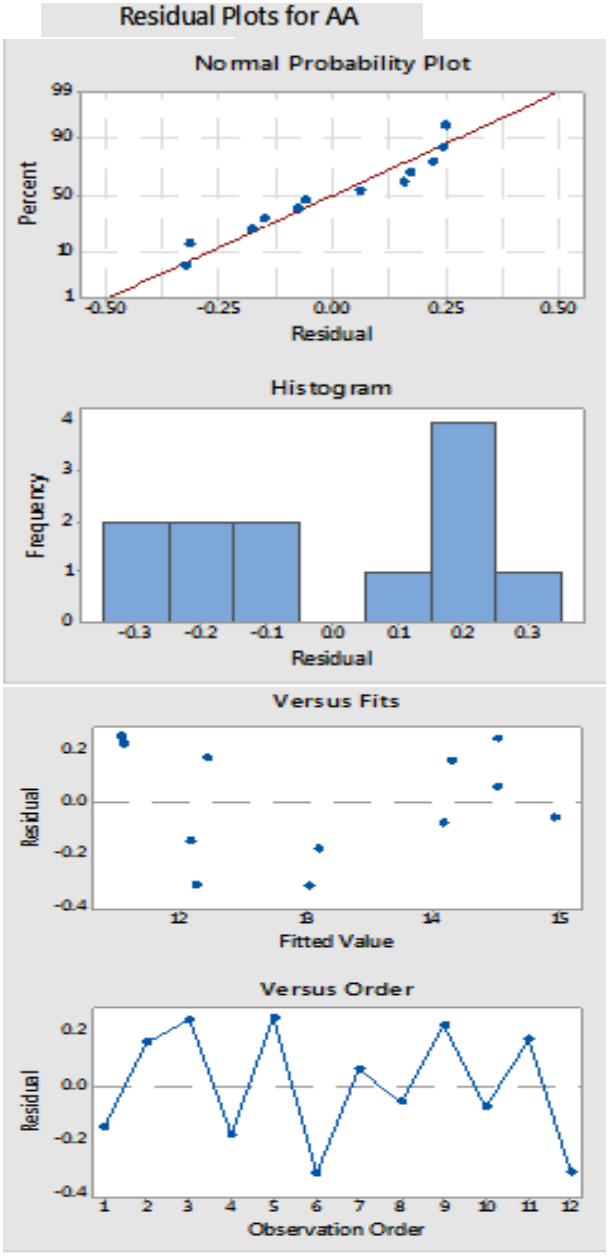

Fig. 2: Residual plots for statistical results of amino acid production by Plackett-Burman

Table 5: Factorial Regression: AA Versus A, B, C, D, E and D

Analysis of Variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Model	6	17.2153	2.8692	28.91	0.001
Linear	6	17.2153	2.8692	28.91	0.001
A	1	0.525	0.525	5.29	0.07
B	1	1.477	1.477	14.88	0.012
C	1	0.0954	0.0954	0.96	0.372
D	1	13.3774	13.3774	134.79	0
E	1	0.6302	0.6302	6.35	0.053
F	1	1.1102	1.1102	11.19	0.02
Error	5	0.4962	0.0992		
Total	11	17.7115			

DF; degree of freedom
Adj: Adjusted

Table 6: Evaluation impact, corresponding P-values and regression coefficient for amino acid production by *Aspergillus flavus* using Plackett-Burman design

Term		Amino Acid Production			
		Effect	**Coefficient***	***P*-value**	**T-Value**
Constant		---	13.1492	0	144.59
A	**Spore suspension**	-0.4183	-0.2092	0.07	-2.3
B	**Incubation period**	0.7017	0.3508	0.012	3.86
C	**pH**	-0.1783	-0.0892	0.372	-0.98
D	**KH_2PO_4**	2.1117	1.0558	0	11.61
E	**$MgSO_4$**	0.4583	0.2292	0.053	2.52
F	**$(NH_4)_2SO_4$**	-0.6083	-0.3042	0.02	-3.34
R^{2*}		97.20%			
Predicted R^2		83.86%			
Adjusted R^2		93.84			

Plackett burmann Regression Equation in Uncoded Units for fungal strain

AA= 10.951 -0.418 A+ 0.1169 B-0.0446 C+0.5279 D+0.458 E-0.507 F

3. Results and Discussion

Discussion

Soybeans are an essential source of low-cost protein. They are widely consumed due to their functionality and nutritive value. Composition of dry weight for soya stalk cellulose 35%, hemicellulose 25%, lignin 20% **[20]**. Recently, the consumption of soybeans has been increasing due to its beneficial effects on human health such as prevention and treatment of various chronic illnesses, which include cardiovascular diseases and various forms of cancer **[21]**.

Microbial proteases are an essential class of enzymes, which constitute more than 65% of the total industrial enzyme market **[22]**. Among different types of fungi *Aspergillus flavus*, *Aspergillus melleu*, *Aspergillus niger*, *Chrysosporium keratinophilum*, *Fusarium graminarum* and *Penicillium griseofulvin* are considered most suitable for SSF **[23,24]**

Proteases are objected from microbial sources, predominantly from extremophiles microbes. **[25]**. SSF display the most favorable environment for fungal growth to maximize productivity in with low-cost process. These techniques carried out by using nutrient-rich agro-industrial residues as substrates. **[26].** According to **[27]** PBD applied for screening purposes, using an indefinite number of experiments to find the best experimental design for survey the dependence of some measured quantity of amino acid produced in matrix from SSF. Six independent variables are tested in SSF conditions each variable has three own levels (low, center and high). The overlapping between variables was negligible. Mathematical analysis of PBD mentioned to the congruent fitted values of free amino acid that were closed to those recorded from the response values (laboratory calculated) these clearly in Fig (2) in residual plots of amino acids. Fitted values were substantial for estimation while the model fits of data referring to the accuracy of the variable selection in **Table** (2).

The probability (*P*) level of tested variables was screened at 0.05 **[27]**. For the production of free amino acids, the *P*-values of the incubation period, KH_2PO_4 and (NH_4) SO_4 were lower than 0.05 with a significant effect, for AA production. R^2 and adjusted R^2 estimated from quiet fitness of the data. Their values help in the selection of an adequated model with the best fit. R^2 coefficient described ratio of variation presented in the observed response values which was mentioned by the factor(s). Adjusted R^2 refers to the modification in R^2 due to the continued rising of R^2 with some additional predictors **[27]**. Increasing in the adjusted R^2 indicated to more accuracy of the relations between the other variables and responses (production of free amino acid). Predicted-R^2 is also like to adjusted R^2 that pointed to how well the model predicts responses for the new observations, while **4.**

adjusted R^2 refers to how well the model fits the data **[27]**. However, all R^2 values range from "0 to 1". Results demonstrated that fungi *Aspergillums flavus* has been the most active proteolytic strain that could degrade proteinaceous residuals of soybean and produced a high amount of free amino acid in the filtrate produced more free amino acids. These results are supported the studies obtained by **[28-31]**.

Conclusion

Enormous amounts of different agricultural wastes are produced worldwide annually. The results obtained in this study showed that time of incubation and the concentration of both KH_2PO_4 and $(NH_4)_2SO_4$ plays an essential role in the production of amino acid under solid-state fermentation conditions. These conditions affect the availability of microbes that

degrading soybean residuals components. In addition to, the superiority of *Aspergillums flavus* over the other tested fungal cultures, for production of free amino acids

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