Research Article

Optimization of Process Parameters by Statistical Experimental Designs for the Production of Naringinase Enzyme by Marine Fungi

Abeer Nasr Shehata¹ and Abeer Abas Abd El Aty²

¹ Biochemistry Department, National Research Centre, Cairo, Egypt

² Chemistry of Natural and Microbial Products Department, National Research Centre, Cairo, Egypt

Correspondence should be addressed to Abeer Abas Abd El Aty; aabass44@yahoo.com

Received 19 September 2013; Revised 10 December 2013; Accepted 10 December 2013; Published 4 February 2014

Academic Editor: Donald L. Feke

Copyright © 2014 A. N. Shehata and A. A. Abd El Aty. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Naringinase has attracted a great deal of attention in recent years due to its hydrolytic activities which include the production of rhamnose and prunin and debittering of citrus fruit juices. Screening of fifteen marine-derived fungi, locally isolated from Ismailia, Egypt, for naringinase enzyme production, indicated that *Aspergillus niger* was the most promising. In solid state fermentation (SSF) of the agroindustrial waste, orange rind was used as a substrate containing naringin. Sequential optimization strategy, based on statistical experimental designs, was employed to enhance the production of the debittering naringinase enzyme. Effects of 19 variables were examined for their significance on naringinase production using Plackett-Burman factorial design. Significant parameters were further investigated using Taguchi's (L16 4⁵) orthogonal array design. Based on statistical analysis (ANOVA), the optimal combinations of the major constituents of media for maximal naringinase production were evaluated as follows: 15 g orange rind waste, 30 mL moisture content, 1% grape fruit, 1% NaNO₃, 0.5% KH₂PO₄, 5 mM MgSO₄, 5 mM FeSO₄, and the initial pH 7.5. The activity obtained was more than 3.14-fold the basal production medium.

1. Introduction

Naringinase, an α -rhamnopyranosidase, expressed as α -L-rhamnosidase (E.C. 3.2.1.40) and β -D-glucosidase (E.C. 3.2.1.21) activities. This hydrolytic enzymatic complex has wide occurrence in nature and has been reported in plants, yeasts, fungi, and bacteria [1]. The former enzyme splits naringin into rhamnose and prunin and the latter further hydrolyses prunin to D-glucose and naringenin, a nonbitter compound, which cannot be converted back to naringin [2, 3].

Naringinase is commercially attractive due to its potential usefulness in pharmaceutical and food industries. It is of particular interest in the biotransformation of steroids and antibiotics and mainly on glycosides hydrolysis. Moreover, it has been used in citrus juices debittering and wine industries [4, 5].

Naringinase is of particular interest to the structure determination of polysaccharides, glycosides, and glycolipids [6].

Naringin and its hydrolyzed product naringenin are useful for inhibiting acyl-coA-cholesterol-*o*-acyltransferase activity, which helps in preventing accumulation of macrophage-lipid complex that causes hepatic diseases [7].

Solid state fermentation (SSF), which has been used infrequently to produce naringinase, is an important tool for the production of industrial fungal enzymes, due to automation capabilities and operating experience with many other large-scale solid-substrate fermentation processes [8–10]. SSF has many advantages over submerged fermentation (SMF), including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery, and lack of foam buildup, and is reported to be the most appropriate process for developing countries. A further advantage of SSF is cheap and easily available substrates, such as agriculture and food industry by-products [11].

In this work, the agroindustrial waste orange rind with high concentration of naringin was utilized as substrate to produce naringinase enzyme, by experimenting with marine-derived fungi as new source in an SSF system, where there is no literature available concerning the production of naringinase by filamentous marine-derived fungi.

The debittering process could be more cost effective and economically viable if naringinase production is achieved industrially using microorganisms. In the present study, an attempt has been made to optimize the process parameters to increase naringinase production.

Statistical experimental designs have been used for several decades and it can be adopted at various phases of an optimization strategy, such as for screening experiments or for looking for the optimal conditions for targeted response(s). Lately, the results analyzed by a statistically planned experiment are better acknowledged than those carried out by the traditional one-variable-at-a-time (OVAT). Some of the popular choices in applying statistical designs to bioprocessing include the Plackett-Burman design [12, 13] and Taguchi experimental design [14].

The Plackett-Burman design provides an efficient way of a large number of variables and identifies the most important ones [15]. Taguchi's experimental design is a fractional factorial experimental design that involves the use of predefined orthogonal arrays to study a maximal number of factors at selected levels with a minimal set of experiments. It allows us to study the influence of individual factors, to establish the interaction between them, and finally to calculate performance at the optimum levels obtained. Additionally, it allows the study of categorical factors (e.g., carbon source type) along with continuous ones (e.g., nitrogen source type). This methodology is applied in many engineering areas and is extensively used to design robust products and processes for industry. Its application in the biotechnology field, in particular in fermentation processes, has also proven to be useful [16].

In the present work, we report for the first time a sequential optimization strategy for naringinase enzyme production by marine-derived local fungal isolate, through statistically designed experiments as an effective tool for medium engineering. First, Plackett-Burman screening design was applied to address the most significant factors affecting enzyme production. Second, Taguchi design was applied to determine the optimum level of each of the significant parameters that brings maximum naringinase production.

2. Materials and Methods

2.1. Microorganism and Culture Media. Fifteen marine-derived filamentous fungi, isolated from decayed wood samples, collected from the old wooden boats submerged in sea water of Ismailia, Egypt, were screened for naringinase production in this study. The Stock cultures were maintained on malt extract agar slants at 4°C and periodically subcultured. The medium composition comprises of biomalt 20 g/L, agar 15 g/l, 800 mL sterile sea water, and 200 mL distilled water [17].

2.2. Solid State Fermentation (SSF). Orange rind was used as substrate for naringinase production. Orange rind was air-dried and cut in small pieces (particle size: 0.96, 1.5, 1.9, and 2.5 mm). Fermentation was carried out in Erlenmeyer flasks (250 mL) with 5 g of orange rind as solid substrate moistened with 10 mL of sea water. The flasks were autoclaved for 20 min at 121°C and inoculated aseptically with 1 mL spore suspension. Incubation was carried out at 28°C, without agitation for 7 days.

2.3. Recovery of the Enzymatic Extract. The extract was recovered using 50 mL of sodium acetate buffer 0.1 M, pH 4.0, added to the solid cultures and placed on a shaker for 30 min. The suspension was filtered through a nylon cloth and then centrifuged at 5,000 rpm for 15 min, 4°C. The filtrate obtained was used for determination of naringinase activity [18].

2.4. Determination of Naringinase Activity. Naringinase was assayed for its activity by measuring the rate of glucose formation from the two steps hydrolysis of naringin to prunin and rhamnose (by naringinase) and of prunin to naringenin and glucose (by β -glucosidase). The reaction mixture, which consists of 0.8 mL of 0.2% naringin solution in 0.1 M sodium acetate buffer, pH 4.0, and 0.2 mL enzyme, was incubated at 50°C for 60 min, after which 1 mL of the reaction mixture was tested for the presence of glucose by DNS method [19]. One unit of naringinase activity was defined as the amount of enzyme that liberates 1 μ mole of reducing sugars, expressed as glucose, under the given assay conditions.

2.5. Statistical Designs

2.5.1. Plackett-Burman (PB) Experimental Design. The Plackett-Burman design was used for screening of the factors (media components) that significantly influenced naringinase production. For screening purpose, various medium components and culture parameters have been evaluated. Based on the Plackett-Burman factorial design, each factor was examined in two levels: (−1) for a low level and (+1) for a high level [20]. This design is practical especially when the investigator is faced with a large number of factors and is unsure which settings are likely to be nearer to optimum responses [21]. Table 1 illustrates the factors under investigation as well as levels of each factor used in the experimental design, whereas Table 2 represents the design matrix.

Plackett-Burman experimental design is based on the first-order polynomial equation:

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

where Y is the response (enzyme activity), β_0 is the model coefficient and β_i is the linear coefficient, and x_i is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. The main

TABLE 1: Media components and test levels for Plackett–Burman experiment.

Variable	Variable code	Low level (−1)	High level (+1)
Moisture content (mL)	A	10	50
Weight of waste (g)	B	5	20
Initial pH	C	4.5	7.5
Glucose (%)	D	1	3
Grape fruit (%)	E	1	3
Sucrose (%)	F	1	3
Starch (%)	G	1	3
Molasses (%)	H	1	3
Peptone (%)	J	0	0.5
Tryptone (%)	K	0	0.5
Yeast extract (%)	L	0	0.5
Soya bean (%)	M	0	0.5
NaNO ₃ (%)	N	0	0.5
(NH ₄) ₂ PO ₄ (%)	O	0	0.5
KH ₂ PO ₄ (%)	P	0.1	0.5
MgSO ₄ (mM)	Q	0	5
MnSO ₄ (mM)	R	0	5
CuSO ₄ (mM)	S	0	5
FeSO ₄ (mM)	T	0	5

Weight of waste (g): orange rind (substrate).

FIGURE 1: Pareto chart representing the percentage contribution of the nutrient components.

effect of each variable was determined according to the following equation:

$$E_{xi} = \frac{(\sum M_{i+} - \sum M_{i-})}{N}, \quad (2)$$

where E_{xi} is the variable main effect, $\sum M_{i+}$ is the summation of the response value at high level, $\sum M_{i-}$ is the summation of

the response value at low level, and N is the number of experiments. In the present work, nineteen assigned variables were screened in twenty experimental designs. All experiments were carried out in duplicate and the averages of naringinase activity were taken as response (Table 2).

2.6. Taguchi Methodology. One-factor-at-a-time [22] and Taguchi methods [14] were used for optimization of culture condition. The optimum incubation period was determined in a separate experiment, by one-variable-at-a-time method. The inoculated flasks were incubated for different time intervals (4, 7, 10, 14, and 18 days). Optimization of five other factors, initial pH, moisture content (mL), weight of grape fruit (%), weight of NaNO₃ (%) as a nitrogen source, and weight of waste (orange rind) (g) as shown in Table 3, was studied by Taguchi method, because it has significant impact on naringinase production as screened by Plackett–Burman design.

To perform the Taguchi method, 16 different experiments, by using L16 orthogonal array, were run, as shown in Table 4. Based on the primary results, a verification test was also performed to check the optimum condition. An analysis of variance (ANOVA) for the obtained results was investigated. For designing the experiments, analysis of variance, and optimization of process, Design-Expert 8 software from Stat-Ease, Inc., was used.

3. Results

3.1. Survey of Some Marine Fungal Isolates for Naringinase Enzyme Production. Fifteen marine-derived fungi were screened for naringinase enzyme production. Results showed that eleven fungal isolates are unable to produce the enzyme. Only four identified as *Aspergillus niger*, *Penicillium nalgiovense*, *Aspergillus flavus*, and *Aspergillus terreus* are capable of producing naringinase enzyme on orange rind substrate (4.42, 4.16, 0.03, and 0.03 U/mL, resp.).

The marine-derived fungus *Aspergillus niger* showed the highest enzyme activity (4.42 U/mL) on the basal medium containing only orange rind and sea water. Also, the fungus *Penicillium nalgiovense* showed high naringinase activity (4.16 U/mL), compared to the other two *Aspergillus* strains.

Out of four positive fungi, the marine-derived fungus *Aspergillus niger* was used in this study.

3.2. Optimization of Naringinase Production by Multifactorial Experiments. Sequential optimization approaches were applied in the present part of the study. The first approach deals with the determination of the optimum incubation period, by one-variable-at-a-time method. The second approach deals with screening for nutritional factors affecting the selected marine-derived fungus *Aspergillus niger* naringinase production. The third approach is to optimize the factors that control the enzyme production process.

3.3. Determination of the Optimum Incubation Period. One-variable-at-a-time method is used to determine the optimum incubation period for naringinase production. Results

TABLE 2: Twenty trials Plackett-Burman experimental design with the response.

Trials	Physical variables			Carbon sources				Nitrogen sources				Energy source				Metal ions				Response enzyme activity (U/mL)	
	Moisture content	Weight of waste	Initial pH	Glucose	Grape fruit	Sucrose	Starch	Molasses	Peptone	Tryptone	Yeast extract	Soya bean	NaNO ₃	(NH ₄) ₂ PO ₄	KH ₂ PO ₄	MgSO ₄	MnSO ₄	CuSO ₄	FeSO ₄	Experimental	Predicted
1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	-1	-1	0.04	0.02
2	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	1.43	1.40
3	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	-1	-1	-1	-1	-1	4.16	4.19
4	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	3.12	3.15
5	-1	+1	+1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	1.56	1.58
6	-1	+1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	-1	-1	+1	-1	+1	0.08	0.09
7	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	1.95	1.97
8	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	3.25	3.22
9	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	0.65	0.63
10	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	-1	+1	-1	+1	3.38	3.36
11	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	-1	-1	-1	-1	-1	+1	+1	-1	2.34	2.31
12	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	2.08	2.06
13	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.26	0.29
14	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	1.69	1.66
15	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	9.10	9.13
16	-1	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	5.72	5.70
17	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	1.63	1.64
18	+1	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	-1	+1	-1	-1	3.51	3.54
19	+1	+1	+1	-1	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	11.70	11.67
20	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1	+1	+1	-1	6.50	6.52

TABLE 3: Factors and their levels which were studied by Taguchi method.

Factor	Level 1	Level 2	Level 3	Level 4
A: initial pH	5.5	6.5	7.5	8.5
B: moisture content (mL)	10	20	30	50
C: weight of grape fruit (%)	1	2	3	5
D: weight of NaNO ₃ (%)	0.5	1	2	3
E: weight of waste (g)	5	10	15	20

indicated that at different incubation periods (4, 7, 10, 14, and 17 days) the activity was (0.15, 4.42, 5.03, 1.37, and 0.46 U/mL, resp.). The incubation period for 10 days was the most favorable for maximum naringinase production where the activity increased about 1.14-fold compared to that at 7 days.

3.4. Evaluation of the Factors Affecting Naringinase Productivity. Nineteen different factors (variables) including fermentation conditions and medium constitution were chosen to perform this optimization process. The averages of naringinase activity for the different trials (experimental) together with the predicted activity from the regression equation (3) for the combinations are shown in Table 2.

The data in Table 2 show a wide variation from 0.04 to 11.70 U/mL of naringinase activity. This variation reflects the importance of medium optimization to attain higher productivity.

The analysis of the data from the Plackett-Burman experiments involved a first-order (main effects) model. The main effects of the examined factors on the enzyme activity were calculated and presented graphically in Figure 2.

On the analysis of the regression coefficients of the 19 variables, initial pH, moisture content, grape fruit, NaNO₃, KH₂PO₄, MnSO₄, weight of waste, FeSO₄, yeast extract, MgSO₄, and CuSO₄ showed positive effect on naringinase activity. Sucrose, molasses, (NH₄)₃PO₄, peptone, tryptone, and glucose were contributed negatively.

The first order model equation developed by PB design showed the dependence of *A. niger* naringinase production on the medium constituents:

Y (naringinase activity U/mL)

$$\begin{aligned}
 &= +3.21 + 1.17 * A + 0.3 * B + 1.63 * C - 0.26 * D \\
 &+ 1.12 * E - 1.07 * F - 0.65 * H - 0.52 * J \\
 &- 0.45 * K + 0.23 * L + 0.51 * N - 0.63 * O + 0.48 * P \\
 &+ 0.20 * Q + 0.40 * R + 0.17 * S + 0.27 * T.
 \end{aligned}
 \tag{3}$$

Statistical analysis of the responses is represented in Table 5. The Model F value of 2231.40 implies that the model is significant. Values of “Prob > F ” less than 0.0500 indicate that model terms are significant. In this case, $B, C, D, E, F, H, J, K, L, N, O, P, Q, R, S$, and T are significant model terms.

In addition, the predicted R^2 was found to be 0.9947, which is in reasonable agreement with the R^2 of 0.9999 and adjusted R^2 of 0.9995. This revealed that there is good agreement between the experimental and the theoretical values predicted by the model and almost all the variation could be accounted for by the model equation.

Overall, the percentage contribution of the significant variables indicated that 30.43% was for initial pH, 15.73% for moisture content, 14.30% for grape fruit, and the remaining 39.54% for the other variables (Figure 1).

The obtained results showed that varying the initial cultivation pH of the medium between pH 4.5 and 7.5 had high effect on naringinase production by the marine-derived *A. niger*. Maximal enzyme activities were obtained only when the initial pH of the culture medium was adjusted to 7.5.

According to these results, a medium of the following composition is expected to be near optimum: 20 g weight of waste, 50 mL moisture content, 3% grape fruit, 0.5% NaNO₃, 0.5% KH₂PO₄, 5 mM MgSO₄, 5 mM FeSO₄, and the initial pH 7.5. The enzyme activity measurement on this medium was 11.70 U/mL. This result presented about 2.33-fold increase in the enzyme activity, when compared to (5.03 U/mL), the results obtained in basal production medium containing only the waste orange rind and sea water after 10 days incubation time.

Thus, the present study indicated that initial pH, moisture content, and grape fruit were found to be the most significant variables affecting naringinase activity. On the other hand, although weight of waste affected the enzyme positively with low rate, it will be preferred in the forthcoming investigation to find out optimum weight of orange rind suitable with the moisture content, for the best solid state fermentation bringing maximum naringinase activity.

Other variables with less significant effect were not included in the next optimization experiment but instead were used in all trials at their (−1) level and (+1) level, for the negatively contributing variables and the positively contributing variables, respectively.

3.5. Optimization of the Culture Conditions by Taguchi Design.

Effects of initial pH, moisture content, weight of grape fruit, NaNO₃, and orange rind waste were studied by Taguchi method, which is a fractional factorial experimental design. Five-four level factors (L16 4⁵) can be positioned in an L16 orthogonal array table. The design and the response enzyme activity (U/mL) are shown in Table 4.

The results of experiments performed in this section showed that the maximum average yield of naringinase was 13.00 U/mL, which occurred when experiment's conditions were as follows: 20 g weight of waste, 30 mL moisture content, 1% grape fruit, 1% NaNO₃, 0.5% KH₂PO₄, 5 mM MgSO₄, 5 mM FeSO₄, and the initial pH 7.5. This result presented about 2.95-fold increase in the enzyme activity, when compared to (4.42 U/mL), the results obtained in the first basal production medium, without any optimization.

Analysis of variance (ANOVA) was applied to the data. The main objective of ANOVA is to extract from the results

TABLE 4: Levels of five different factors, applied in each of 16 trials, and the obtained results.

Trial number	Factor 1 A: Initial pH	Factor 2 B: Moisture content (mL)	Factor 3 C: Weight of grape fruit (%)	Factor 4 D: Weight of NaNO ₃ (%)	Factor 5 E: Weight of waste (g)	Response enzyme activity (U/mL)
1	1	4	4	4	4	0.00
2	2	4	3	2	1	5.46
3	2	1	2	3	4	0.00
4	4	2	3	1	4	6.76
5	3	3	1	2	4	13.00
6	1	1	1	1	1	6.50
7	1	2	2	2	2	0.00
8	3	4	2	1	3	7.54
9	3	2	4	3	1	6.50
10	4	4	1	3	2	5.98
11	2	2	1	4	3	6.76
12	3	1	3	4	2	8.06
13	1	3	3	3	3	8.32
14	2	3	4	1	2	0.00
15	4	1	4	2	3	0.00
16	4	3	2	4	1	5.72

The numbers under factors refer to their levels; for description of levels, refer to Table 3.

TABLE 5: Statistical analysis (ANOVA) for evaluating the significance of variables.

Source	Sum of squares	F value	P value Prob > F
Model	174.37	2231.40	0.0004 significant
A: moisture content	27.44	5969.12	0.0002
B: weight of waste	2.08	452.24	0.0022
C: initial pH	53.07	11544.18	<0.0001
D: glucose	1.38	300.03	0.0033
E: grape fruit	24.94	5425.60	0.0002
F: sucrose	22.89	4980.36	0.0002
H: molasses	8.48	1845.60	0.0005
J: peptone	5.35	1164.74	0.0009
K: tryptone	4.00	870.12	0.0011
L: yeast extract	1.07	232.97	0.0043
N: NaNO ₃	5.19	1129.88	0.0009
O: (NH ₄) ₂ PO ₄	7.89	1715.36	0.0006
P: KH ₂ PO ₄	4.55	990.36	0.0010
Q: MgSO ₄	0.82	178.94	0.0055
R: MnSO ₄	3.23	702.07	0.0014
S: CuSO ₄	0.58	126.18	0.0078
T: FeSO ₄	1.41	306.00	0.0033

$R^2 = 0.9999$; adjusted $R^2 = 0.9995$; predicted $R^2 = 0.9947$; adequate precision = 181.134; cumulative variance (C.V.%) = 2.11.

how much variations each factor cause relative to the total variation observed in the result. From the results of ANOVA

TABLE 6: Analysis of variance (ANOVA) of main effects of factors.

Source	Sum of squares	df	Fvalue	P value Prob > F
Model	227.24	12	58.97	0.0032 significant
Initial pH	79.41	3	82.44	0.0022
Moisture content (mL)	20.03	3	20.79	0.0165
Weight of grape fruit (%)	112.84	3	117.14	0.0013
Weight of waste (g)	14.96	3	15.53	0.0248
Residual	0.96	3		
Cor Total	228.20	15		

Std. Dev. 0.57; Mean = 5.04; C.V.% = 11.25; PRESS = 27.40; $R^2 = 0.9958$; Adj $R^2 = 0.9789$; Pred $R^2 = 0.8799$; Adeq Precision = 26.597.

in Table 6, we found that changing the initial pH, moisture content, weight of powdered grape fruit, and orange rind waste of medium is the most important factor in causing differences in obtained results. By the same way, the least important factor was the nitrogen source.

Analysis of variance (ANOVA) of main effects of factors indicated that the model F value of 58.97 implies that the model is significant. There is only a 0.32% chance that a "Model F Value" this large could occur due to noise. Values of "Prob > F " less than 0.0500 indicate that model terms are significant. In this case A, B, C, and E are significant model terms. Represent initial pH, moisture content, weight of powdered grape fruit and weight of orange rind waste, respectively. And The "Pred R -Squared" of 0.8799 is in reasonable agreement with the "Adj R -Squared" of 0.9789.

TABLE 7: Optimum conditions suggested by statistical calculations after performing the tests.

Factor	Name	Level	Low Level	High Level
A	Initial pH	7.5	5.5	8.5
B	Moisture content	30	10	50
C	G. fruit	1	1	5
D	NaNO ₃	1	0.5	3
E	Weight of waste	15	5	20

99% of Population										
Response	Prediction	Std dev	SE mean	95% CI low	95% CI high	SE pred	95% PI low	95% PI high	95% TI low	95%TI high
E. activity	14.1375	0.566657	0.510778	12.512	15.763	0.762885	11.7097	16.565	8.67337	19.6016

FIGURE 2: Main effects of the medium constituents on *A. niger* naringinase production by the Plackett-Burman experimental results.

Final equation in terms of coded factors is

$$\begin{aligned}
 Y_{\text{activity}} = & + 5.04 - 1.33 * A [1] - 1.98 * A [2] + 3.74 * A [3] \\
 & - 1.40 * B [1] - 0.033 * B [2] + 1.72 * B [3] \\
 & + 3.02 * C [1] - 1.72 * C [2] + 2.1 * C [3] \\
 & + 1.01 * E [1] - 1.53 * E [2] + 0.62 * E [3].
 \end{aligned}
 \quad (4)$$

FIGURE 3: Predicted response versus actual value.

The above model can be used to predict the naringinase production within the limits of the experimental factors. Figure 3 Shows that the actual response values agree well with the predicted response values. The interaction effects of variables on naringinase production were studied by plotting 3D surface plot against any two independent variables, as in Figures 4 and 5.

When these results were analyzed, an optimum condition was suggested by calculations. Table 7 shows the suggested condition. Statistical calculations predicted that, if the conditions were chosen as shown in Table 7, the enzyme production should reach 14.14 U/mL. However, after performing the fermentation at said condition, the produced naringinase was about 13.89 U/mL, but since the difference between predicted and actual result was only about 1.77%, it should be regarded as acceptable. Therefore, comparing with 13 U/mL, produced before, a further increase of about 1.07-fold was achieved. This reflects the necessity and value of optimization process.

4. Discussion

The marine-derived fungus *Aspergillus niger* showed the highest enzyme activity on the basal medium, compared to

FIGURE 4: 3D surface plot showing the effect of initial pH and moisture content on naringinase activity.

FIGURE 5: 3D surface plot showing the effect of initial pH and weight of waste on naringinase activity.

the other two *Aspergillus* sp. Also, the fungus *Penicillium nalgioense* showed high naringinase activity. Although fungi of the genera *Penicillium* have been reported as producers of this enzyme [23–26], *Aspergillus* genus had been used preferentially for enzyme production for a number of years. This bias is due to its high level of extracellular enzyme production seen in this genus.

In recent years the enzyme production industry (biotech) has encountered problems with undesirable levels of carbohydrate content in the synthetic medium utilized in reduced-bitter enzyme production. The presence of those substances

posses an inhibitory effect on naringinase production. Some researchers have used media incorporating naringin in order to increase enzyme production in an SSF system. In this work we elucidate the suitability and advantages of using orange rind, an abundant and economical agricultural waste product, as a substrate for naringinase production.

One-variable-at-a-time method is used to determine the optimum incubation period for naringinase production. The results showed that the incubation period for 10 days was the most favorable for maximum *A. niger* naringinase production. These results one nearly similar to those reported by [27] who found that the maximal naringinase activity was obtained at a longer incubation time (9 days) by *Aspergillus niger* MTCC 1344.

For screening purpose, various medium components as well as environmental factors were evaluated by Plackett-Burman design. PB design offers a good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which saves time and maintains convincing information on each component [13].

On the analysis of the regression coefficients of the 19 variables, initial pH, moisture content, grape fruit, NaNO_3 , KH_2PO_4 , MnSO_4 , weight of waste, FeSO_4 , yeast extract, MgSO_4 , and CuSO_4 showed positive effect on naringinase activity. Sucrose, molasses, $(\text{NH}_4)_2\text{PO}_4$, peptone, tryptone and glucose were contributed negatively.

Maximal enzyme activities were obtained only when the initial pH of the culture medium was adjusted to 7.5. This is a logic observation because the culture used in the present study was isolated from marine source where the pH lied in the neutral to alkaline range. Neutral to alkaline pH was observed to be most favourable condition for naringinase production by this strain than the highly acidic conditions. This lind with Puri et al., 2005 [27] who found that the Maximal *Aspergillus niger* MTCC 1344 naringinase activity was obtained when the initial pH of the culture medium was adjusted to 4.5.

It appears that the source of isolation plays an important role in obtaining isolates with desired traits. Geofungi growing in marine environments appear to be conditioned to their environment [28]. Therefore, *A. niger* appears to be a good candidate for biotechnological application in alkaline conditions.

Moisture content of the culture medium is considered an important factor affecting the enzyme production in solid state fermentation, performed on a nonsoluble material in the absence of free flowing liquid. [29]. This is in agreement with the findings of this work, since the moisture content was found to contribute significantly to the naringinase production.

As the microorganisms in SSF are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation [30, 31].

It is not surprising that grape fruit powder appeared to be one of the most effective variables during naringinase production by *A. niger* with high percent contribution; this

is because it can serve as a carbon source and an inducer. Grape fruit powder is a waste of fruit process, which means that it is a cheap resource and has additional environmental benefits. These results agreed with [32] who found that grape fruit powder was the most suitable carbon source for maximal naringinase production by *Aspergillus oryzae* JMU316.

The type of nitrogen source which influences the production levels of naringinase was the inorganic nitrogen source NaNO_3 . This agrees with [33] that stated that the maximum naringinase production of *Aspergillus niger* BCC 25166 obtained by supplement of the medium with NaNO_3 as its nitrogen source. But in another study peptone proved to be the most suitable nitrogen source for naringinase production by *Aspergillus oryzae* JMU316 [32].

Statistical methods have been applied for optimization of microbial enzyme production in various studies [34–37]. The use of a good reliable statistical model is essential to develop better strategies for the optimization of the fermentation process [38]. In this regard, Taguchi approach of orthogonal array experimental design for bioprocess optimization is a good reliable statistical model. [39] successfully applied Taguchi experimental design for lipase production by *Bacillus* F3 and observed enhanced production.

In this study, Taguchi design was successfully applied to test the relative importance of medium components and environmental factors on naringinase production. The maximum naringinase production of 13.89 U/mL was achieved under optimal experimental conditions. This result would further facilitate economic design of the large-scale fermentation operation system.

5. Conclusions

In this work, the applied statistical tools proved to be efficient for optimizing naringinase enzyme production in solid state fermentation. Plackett-Burman design was used to test the relative importance of medium components on naringinase production. Among the variables, initial pH, moisture content, and grape fruit were found to be the most significant variables. From further optimization studies, using Taguchi method, the optimized values of the variables for naringinase production were as follows: 15 g orange rind waste, 30 mL moisture content, 1% grape fruit, 1% NaNO_3 , 0.5% KH_2PO_4 , 5 mM MgSO_4 , 5 mM FeSO_4 , and the initial pH 7.5. Using the optimized conditions, the experimentally obtained naringinase activity reaches 13.89 U/mL. The results show a close concordance between the expected and obtained activity level. This study showed that naringinase production by the marine-derived *Aspergillus niger* strain could be enhanced by statistical optimization of the medium components using the cheaper substrate orange rind.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] M. H. L. Ribeiro, "Naringinases: occurrence, characteristics, and applications," *Applied Microbiology and Biotechnology*, vol. 90, no. 6, pp. 1883–1895, 2011.
- [2] M. Ono, T. Tosa, and I. Chibata, "Preparation and properties of immobilized naringinase using tannin aminoethyl cellulose," *Agricultural and Biological Chemistry*, vol. 43, pp. 1847–1851, 1978.
- [3] D. W. Thomas, C. V. Smythe, and M. D. Labbee, "Enzymatic hydrolysis of naringin, the bitter principle of grapefruit," *Food Research*, vol. 23, no. 6, pp. 591–598, 1958.
- [4] M. H. L. Ribeiro, C. Afonso, H. J. Vila-Real, A. J. Alfaia, and L. Ferreira, "Contribution of response surface methodology to the modeling of naringin hydrolysis by naringinase Ca-alginate beads under high pressure," *LWT—Food Science and Technology*, vol. 43, no. 3, pp. 482–487, 2010.
- [5] H. Vila-Real, A. J. Alfaia, M. E. Rosa, A. R. Calado, and M. H. L. Ribeiro, "An innovative sol-gel naringinase bioencapsulation process for glycosides hydrolysis," *Process Biochemistry*, vol. 45, no. 6, pp. 841–850, 2010.
- [6] S. Kamiya, S. Esaki, and R. Tanaka, "Synthesis of some disaccharides containing an L-rhamnopyranosyl or L-mannopyranosyl residue, and the substrate specificity of α L-rhamnosidase from *Aspergillus niger*," *Agricultural and Biological Chemistry*, vol. 49, no. 1, pp. 55–62, 1985.
- [7] S. Bok, T. Jeong, and K. H. Bae, "Naringin and naringenin as inhibitors of acyl coA-cholesterol-o-acyltransferase," US Patent 6(165):984, 2000.
- [8] Y. Chisti, "Solid substrate fermentations, enzyme production, food enrichment," in *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, M. C. Flickinger and S. W. Drew, Eds., vol. 5, pp. 2446–2462, John Wiley & Sons, New York, NY, USA, 1999.
- [9] A. Pandey, P. Selvakumar, C. R. Soccol, and P. Nigam, "Solid state fermentation for the production of industrial enzymes," *Current Science*, vol. 77, no. 1, pp. 149–162, 1999.
- [10] L. P. Ooijkaas, F. J. Weber, R. M. Buitelaar, J. Tramper, and A. Rinzema, "Defined media and inert supports: their potential as solid-state fermentation production systems," *Trends in Biotechnology*, vol. 18, no. 8, pp. 356–360, 2000.
- [11] M. Stredansky, E. Conti, L. Navarini, and C. Bertocchi, "Production of bacterial exopolysaccharides by solid substrate fermentation," *Process Biochemistry*, vol. 34, no. 1, pp. 11–16, 1999.
- [12] C. Liu, Y. Liu, W. Liao, Z. Wen, and S. Chen, "Application of statistically-based experimental designs for the optimization of nisin production from whey," *Biotechnology Letters*, vol. 25, no. 11, pp. 877–882, 2003.
- [13] Y. R. Abdel-Fattah, H. M. Saeed, Y. M. Gohar, and M. A. El-Baz, "Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs," *Process Biochemistry*, vol. 40, no. 5, pp. 1707–1714, 2005.
- [14] R. K. Roy, *Design of Experiments Using the Taguchi Approach*, Wiley-Interscience, New York, NY, USA, 2001.
- [15] D. V. Deshmukh and P. R. Puranik, "Application of plackett-burman design to evaluate media components affecting antibacterial activity of Alkaliphilic Cyanobacteria isolated from Lonar lake," *Turkish Journal of Biochemistry*, vol. 35, no. 2, pp. 114–120, 2010.
- [16] R. S. Rao, C. G. Kumar, R. S. Prakasham, and P. J. Hobbs, "The Taguchi methodology as a statistical tool for biotechnological

- applications: a critical appraisal," *Biotechnology Journal*, vol. 3, no. 4, pp. 510–523, 2008.
- [17] U. Höller, G. M. König, and A. D. Wright, "A new tyrosine kinase inhibitor from a marine isolate of *Ulocladium botrytis* and new metabolites from the marine fungi *Asteromyces cruciatus* and *Varicosporina ramulosa*," *European Journal of Organic Chemistry*, no. 11, pp. 2949–2955, 1999.
 - [18] A. Mendoza-Cal, L. Cuevas-Glory, G. Lizama-Uc, and E. Ortiz-Vázquez, "Naringinase production from filamentous fungi using grapefruit rind in solid state fermentation," *African Journal of Microbiology Research*, vol. 4, no. 19, pp. 1964–1969, 2010.
 - [19] G. L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry*, vol. 31, no. 3, pp. 426–428, 1959.
 - [20] R. L. Plackett and J. P. Burman, "The design of optimum multifactorial experiments," *Biometrika*, vol. 33, no. 4, pp. 305–325, 1946.
 - [21] R. Strobel and G. Sullivan, "Experimental design for improvement of fermentations," in *Manual of Industrial Microbiology and Biotechnology*, A. L. Demain and J. E. Davies, Eds., pp. 80–93, ASM Press, Washington, DC, USA, 1999.
 - [22] D. P. Haaland, *Experimental Design in Biotechnology*, Marcel Dekker, New York, NY, USA, 1989.
 - [23] F. Michon, V. Pozsgay, J. Brisson, and H. J. Jennings, "Substrate specificity of naringinase, an α -l-rhamnosidase from *Penicillium decumbens*," *Carbohydrate Research*, vol. 194, pp. 321–324, 1989.
 - [24] D. Cowan, "Industrial enzymes in biotechnology," in *The Science and the Business*, V. Moses and R. Cape, Eds., pp. 311–340, Horwood Academic Publishers, London, UK, 1991.
 - [25] H. Tsen and G. Yu, "Limonin and naringin removal from grapefruit juice with naringinase entrapped in cellulose triacetate fibers," *Journal of Food Science*, vol. 56, no. 1, pp. 31–34, 1991.
 - [26] S. Elinbaum, H. Ferreyra, G. Ellenrieder, and C. Cuevas, "Production of *Aspergillus terreus* β -L-rhamnosidase by solid state fermentation," *Letters in Applied Microbiology*, vol. 34, no. 1, pp. 67–71, 2002.
 - [27] M. Puri, A. Banerjee, and U. C. Banerjee, "Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344," *Process Biochemistry*, vol. 40, no. 1, pp. 195–201, 2005.
 - [28] C. Raghukumar and S. Raghukumar, "Barotolerance of fungi isolated from deep-sea sediments of the Indian Ocean," *Aquatic Microbial Ecology*, vol. 15, no. 2, pp. 153–163, 1998.
 - [29] A. Pandey, "Recent process developments in solid-state fermentation," *Process Biochemistry*, vol. 27, no. 2, pp. 109–117, 1992.
 - [30] B. Han, J. L. Kiers, and R. M. J. Nout, "Solid-substrate fermentation of soybeans with *Rhizopus spp.*: comparison of discontinuous rotation with stationary bed fermentation," *Journal of Bioscience and Bioengineering*, vol. 88, no. 2, pp. 205–209, 1999.
 - [31] A. P. Goes and J. D. Sheppard, "Effect of surfactants on α -amylase production in a solid substrate fermentation process," *Journal of Chemical Technology and Biotechnology*, vol. 74, no. 7, pp. 709–712, 1999.
 - [32] D. Chen, T. Niu, and H. Cai, "Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316," *African Journal of Biotechnology*, vol. 9, no. 31, pp. 4970–4978, 2010.
 - [33] K. Thammawat, P. Pongtanya, V. Junthasasri, and P. Wongvit-hoonyaporn, "Isolation, preliminary enzyme characterization and optimization of culture parameters for production of naringinase isolated from *Aspergillus niger* BCC 25166," *Kasetsart Journal (Natural Science)*, vol. 42, no. 1, pp. 61–72, 2008.
 - [34] G. Dey, A. Mitra, R. Banerjee, and B. R. Maiti, "Enhanced production of amylase by optimization of nutritional constituents using response surface methodology," *Biochemical Engineering Journal*, vol. 7, no. 3, pp. 227–231, 2001.
 - [35] F. Francis, A. Sabu, K. M. Nampoothiri, G. Szakacs, and A. Pandey, "Synthesis of α -amylase by *Aspergillus oryzae* solid-state fermentation," *Journal of Basic Microbiology*, vol. 42, no. 5, pp. 320–326, 2002.
 - [36] S. K. Ahuja, G. M. Ferreira, and A. R. Moreira, "Application of Plackett-Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium," *Biotechnology and Bioengineering*, vol. 85, no. 6, pp. 666–675, 2004.
 - [37] A. Kunamneni, K. S. Kumar, and S. Singh, "Response surface methodological approach to optimize the nutritional parameters for enhanced production of α -amylase in solid state fermentation by *Thermomyces lanuginosus*," *African Journal of Biotechnology*, vol. 4, no. 7, pp. 708–716, 2005.
 - [38] A. E. Ghaly, M. Kamal, and L. R. Correia, "Kinetic modelling of continuous submerged fermentation of cheese whey for single cell protein production," *Bioresource Technology*, vol. 96, no. 10, pp. 1143–1152, 2005.
 - [39] K. M. Heravi, F. Eftekhari, B. Yakhchali, and F. Tabandeh, "Isolation and identification of a lipase producing *Bacillus* sp. from soil," *Pakistan Journal of Biological Sciences*, vol. 11, no. 5, pp. 740–745, 2008.