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Original Research Article

Optimization of naringinase production from *Aspergillus flavus* in solid state fermentation media using citrus peel as support

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Abstract: Naringinase is an enzyme complex, contains rhamnosidase and glucosidase. It is commercially attractive due to its potential usefulness in pharmaceuticals and food industries. Statistical experimental designs can be adopted at various phases of optimization to increase the enzyme production. In the first step of optimization using Plackett-Burman design, naringin, sucrose, NH_4NO_3 and citrus peel significantly affected the naringinase activity of *Aspergillus flavus*. In the second step, L9 (3^4) orthogonal design was applied to determine the optimal concentration of each significant variable. The optimum values for the significant variables were obtained as follows: 10 g/L naringin, 7.5 g/L citrus peel, 5 g/L NH_4NO_3 and 5g/L sucrose. Under this optimum condition, the naringinase activity increased up to 1480.96 ± 13.998 U/g dry matter. This activity was further increased up to 2346.48 U/g dry matter when the culture condition were optimized. Highest activity was obtained at 25ºC and pH 5.0 on the 6thday of fermentation. Optimization of culture conditions and the solid fermentation media resulted in 3.74% increase in the naringinase production from *Aspergillus flavus*. Optimization of media composition and culture conditions by statistical design is efficient to increase the naringinase production by *Aspergillus flavus* in solid media. Optimization of solid stage fermentation system using paddy husk as substrate facilitates the utilization of agro waste. This study concludes that the usage of citrus peel significantly increases naringinase activity of *Aspergillus flavus*. Further this method eliminates the issue of waste disposal in citrus fruit processing industries.
Keywords: *Aspergillus flavus*, factorial experiment, naringinase, optimization, orthogonal contrast, Plackett-Burman design

INTRODUCTION

Naringinase is an enzyme complex composed of two different enzymes rhamnosidase and glucosidase. It hydrolyses naringin in a two-step reaction where rhamnosidase first splits the naringin to prunin (aglycone and D-glucose) liberating one molecule of L-rhamnose and glucosidase hydrolyses prunin into non-bitter naringenin, liberating one molecule of D-glucose [1, 2]. Naringinase is commercially attractive due to its potential usefulness in pharmaceuticals and food industries [6]. Hydrolyzed product prunin has anti-inflammatory and antiviral activity [3], rhamnosidase could be used in the preparation of food additives from biopolymers and preparation of sweeteners [4] and debittering of citrus juice [5]. *Aspergillus flavus* is an efficient producer of α-amylase [7], lipase [8], xylanse [9] and naringinase [10]. Solid stage fermentation found to cost effective and environmental benefit method. In this work paddy husk was used as substrate, which consider as agro waste and citrus peel was used as one of media composition, which consider as industrial waste. This will eliminate waste disposal problem. Statistical experiment designs can be adopted at various phases of optimization, such as screening of important factors and for find out optimum condition. Placket-Burman design helps to find influencing factors and minimize wastage of time and money [11]. Orthogonal contrast method is fractional factorial experiment, which is used to investigate the relationships among various medium components and to optimize their concentrations. Factorial experiment is used find out main effect with interaction effect and optimum level of each factor. Therefore the objective of this study was to screen the significant variables by Placket-Burman design, to find out optimum level of each factor by orthogonal matrix method and to determine the optimum conditions as well as the main effect of each level with interaction effect in solid state fermentation to increase naringinase activity in cost effective way.

MATERIALS AND METHODS

Chemicals

Naringin was obtained from Sigma, St. Louis, USA. All other reagents were in analytical grade. The chemicals used were from standard sources.

Microorganism for enzyme production

Naringinase from *Aspergillus flavus* previously isolated from decaying citrus fruitwas used in this study.

Naringinase assay

Crude enzyme and substrate were pre incubated at 35ºC for 3 minutes. After that pre incubated 0.25ml of crude enzyme was added to 0.25ml pre incubated substrate that was naringin. Here substrate concentration was 1%, pH was 4.5 and incubation time was 10 minutes and temperature was 45ºC. After predetermined reaction condition, the reaction was stopped by boil for 5 minutes after addition of DNS acid [18]. Finally it was allowed to cool and final volume was made up to 6ml with distilled water. Absorbance of test was measured with the help of blank by spectrometer. Blank was prepared by add 0.5 ml DNS acid to the 0.25ml substrate and stirred well, then add the 0.25ml supernatant and followed the procedure same as test.

Medium optimization with statistically-based experimental designs (Plackett-Burman Design and orthogonal contrast method, general full factorial experiment)

Screening important media components through Plackett-Burman(PB)design

A Plackett-Burman design was used to identify the important ingredients of the medium, which have significant influence on naringinase activity. This design does not consider interaction effects among factors, but screening most important factors and remove the dispensable ones to get a smaller and more manageable set of factors. Here this design was used to screen the important C and N sources (factors) for naringinase activity. For that different factors were prepared in two levels, -1 for low level and +1 for high level based on Placket-Burman design (Table 1). The design matrix (Table 4) was developed using Minitab software (Version 13.1, Minitab Co., PA, USA). Solid state fermentation was carried out in 100 ml conical flask with 25 ml of medium and 20% occupied by paddy husk. 6 days old $1*10^5$ spores were inoculated to fermentation medium. The fermentation flasks were kept at room temperature in dark incubator for 8 days. After 8 days the naringinase activity was measured under standard assay condition.

Table-1: Factors (media components) and test levels in Plackett-BurmanDesign

No	Factors	High level (+1)	Low level (-1)
1	Citrus peel (g/L)	10	5
2	NH_4NO_3 (g/L)	10	5
3	Naringin (g/L)	10	5
4	Peptone (g/L)	10	5
5	Sucrose (g/L)	10	5
6	Glucose (g/L)	10	5
7	Soya bean extract (g/L)	10	5

Optimization of media components by orthogonal matrix method

The orthogonal matrix L9 (3^4) method was used to investigate the relationships among various medium components; those were selected by PB design and to optimize their concentrations for naringinase production. Factors and their levels which were studied by orthogonal matrix method presented in Table 2. Other than above levels of factors fermentation conditions, spore size and spore age were maintained same as PB design.

Table-2: Factors and their levels which were studied by orthogonal matrix method

No	Factors	Level 1	Level 2	Level 3
1	Citrus peel (g/L)	5	7.5	10
2	NH_4NO_3 (g/L)	5	7.5	10
3	Naringin (g/L)	5	7.5	10
4	Sucrose (g/L)	5	7.5	10

Optimization of culture conditions

Further naringinase activity was increased by optimize culture conditions such as temperature, pH and fermentation time through full general factorial experiments (Table 7). The medium which was gave highest activity through orthogonal contrast method were used for condition optimization.

Table-3: Factors and their levels which were studied by general full factorial experiment.

Factors	Levels of factors						
	1	2	3	4	5	6	7
Temperature(ºC)	25	32	35	37	-	-	-
pH	4.0	4.5	5.0	5.5	6.0	-	-
Fermentation time (days)	3	4	5	6	7	8	9

RESULTS

Screening important medium components through PB design

First optimization step was carried out by 12 run PB design to screen out significant factors which increase naringinase activity by *Aspergillus flavus*. According to resulting effects of seven variables associated with significant levels through PB design presented in Table 3 and figure 1, revealed increasing concentration of naringin, NH_4NO_3, sucrose and citrus peel have positive effect on naringinase activity within tested limits (Table 1). With the help of relative ranking of effect (Table 4) naringin, NH_4NO_3, sucrose and citrus peel within the tested limits were selected for further optimization by orthogonal contrast method.

Table-4: The Experimental Design Using Plackett-Burman Design for Screening of important Medium Components

Run	Variables							
	Citrus peel	NH_4NO_3	Naringin	Peptone	Sucrose	Glucose	Soya bean extract	Activity (U/g)
1	1	1	1	-1	1	1	-1	705.247
2	-1	1	-1	-1	-1	1	1	20.234
3	1	-1	1	1	-1	1	-1	237.200
4	1	-1	-1	-1	-1	-1	-1	26.800
5	-1	1	1	-1	1	-1	-1	664.250
6	1	1	-1	1	1	-1	1	180.300
7	-1	1	1	1	-1	1	1	98.061
8	-1	-1	1	1	1	-1	1	76.384
9	-1	-1	-1	1	1	1	-1	47.800
10	1	-1	-1	-1	1	1	1	150.897
11	1	1	-1	1	-1	-1	-1	197.500
12	1	-1	1	-1	-1	-1	1	159.592

The arrangement of columns and rows was decided by Plackett-Burman design with the help of Minitab software (Version 13.1, Minitab Co., PA, USA). 1 and -1 represent two concentrations for each factor as same as that in Table-1.

Table-5: Estimated effects, Coefficients, T Values and Significance Levels Calculated from the Naringinase Activity Obtained in the Screening Experiments of PB Design

Term	Effect	Coefficient	t value	P
Citrus peel	116.20	58.10	6.40	0.003**
NH_4NO_3	194.49	97.24	10.72	0.000**
Naringin	219.53	109.77	12.10	0.000**
Peptone	-148.30	-74.15	-8.17	0.001
Sucrose	180.92	90.46	9.97	0.001**
Glucose	-7.56	-3.78	-0.42	0.698
Soya bean extract	-198.89	-99.44	-10.96	0.000

**P<0.05 (significant for a 95% confidence level)

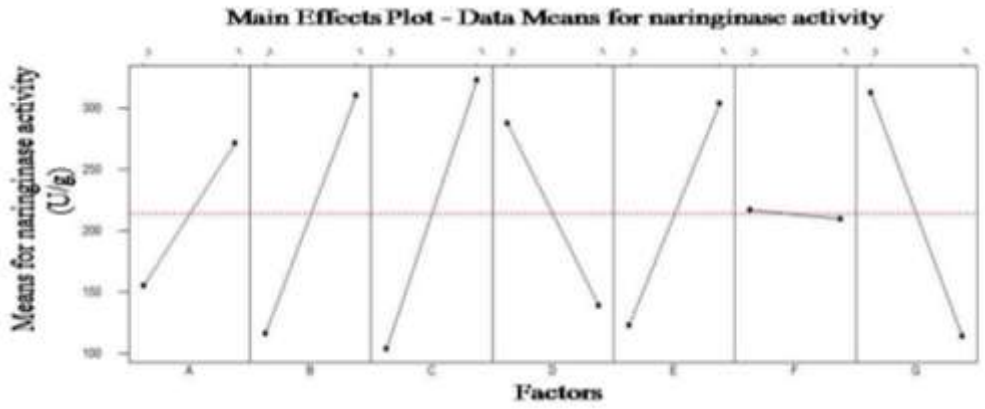

Fig-1: Main effects plot of different factors, which were used as components of medium for optimize naringinase activity. Here A- Citrus peel, B-NH_4NO_3, C-Naringin, D-Peptone, E-Sucrose, F-Glucose, and G- Soya bean extract. 1 and -1 represent levels of each factor.

Optimization of medium components by orthogonal matrix method

From PB design naringin, citrus peel, NH_4NO_3 and sucrose were selected to optimize naringinase activity orthogonally. The optimum activity was obtained where medium composition was 10 g/L naringin, 7.5 g/L citrus peel, 5 g/L NH_4NO_3 and 5g/L sucrose.

Table-6: Results of L9 (3^4) orthogonal test of naringinase production by *Aspergillus flavus* in solid state fermentation.

Exp. group	C	A	B	E	Naringinase activity (U/g)
1	1	1	1	1	566.13 ± 4.646
2	1	2	2	2	904.47 ± 11.983
3	1	3	3	3	1199.18 ± 2.273
4	2	1	2	3	1210.03 ± 14.318
5	2	2	3	1	1121.80 ± 10.472
6	2	3	1	2	1199.85 ± 9.555
7	3	1	3	2	1299.51 ± 23.574
8	3	2	1	3	1480.96 ± 13.998
9	3	3	2	1	1364.85 ± 14.779

Values are mean ± S.D. of triple determinations. Each row of the experimental group number represents one experimental replicate, and every experimental group was replicated thrice. The arrangement of columns was decided by orthogonal design for L9 (34). Numbers 1–3 represent three concentrations for each factor as same as that in Table-2.

Optimization of culture conditions

The ANOVA table (Table 8) obtained from factorial experiment showed ($P<0.05$) the temperature, pH and fermentation days significantly affect the naringinase activity. The results obtained from factorial experiment represented Table 7 and Figure 2 revealed the optimum activity was obtained at 25ºC, pH 5 and 6 days fermentation. Factorial experiment not only expresses main effect but also expresses interaction effect. Interaction effect represented in Figure 3 revealed the effect of one level of factor affect expression of another level of factor. So this experiment facilitates correct identification of level of each level of factors to give highest activity.

Table-7: Results of factorial experiment of medium conditions on naringinase production by *Aspergillus flavus* in solid state fermentation

Run order	H	I	J	Naringinase activity (U/g)
1	2	1	5	1240.04
2	1	3	5	2015.15
3	4	3	2	674.30
4	1	3	1	899.06
5	3	5	5	755.68
6	4	3	3	840.94
7	4	2	3	676.24
8	4	4	5	796.37
9	2	3	2	1290.42
10	4	3	4	852.56
11	2	5	1	434.01
12	2	1	4	1307.86
13	4	2	1	515.41
14	2	4	1	819.59
15	3	5	2	821.56
16	1	5	5	1654.74
17	3	2	4	1362.16
18	1	5	6	1507.48
19	4	2	4	848.69
20	4	3	6	571.60
21	4	3	7	476.66
22	3	4	2	924.26
23	3	5	3	1104.46
24	4	1	5	800.25
25	1	3	7	1743.88
26	3	3	1	618.11
27	1	2	7	1325.35
28	4	4	3	664.61
29	3	3	4	1433.86
30	1	3	3	1926.83
31	2	2	4	1393.11
32	1	2	2	831.24
33	2	2	3	676.24
34	4	5	2	426.28
35	2	2	2	1154.79
36	1	1	4	1242.02
37	1	1	1	145.32
38	3	4	1	569.67
39	2	3	5	2042.19
40	2	4	4	1875.56
41	3	2	6	1038.58
42	3	1	4	1261.41
43	3	4	3	1276.91
44	2	3	7	1689.56
45	2	1	7	1077.29
46	3	3	5	889.38
47	2	3	4	2139.07
48	4	2	5	724.68
49	4	3	1	457.28
50	4	1	7	581.29
51	4	5	7	379.78
52	4	5	5	767.31
53	1	5	2	1026.95
54	1	1	2	567.73
55	2	2	5	1362.11
56	1	1	3	777.00
57	3	2	5	1232.34
58	4	5	3	639.42
59	3	1	3	1145.15
60	2	5	5	1534.55
61	1	1	7	819.62
62	1	4	2	1114.15
63	1	5	3	1240.09
64	1	5	1	538.66
65	2	2	7	1232.29
66	4	4	4	943.63
67	1	2	5	1712.87
68	1	2	6	1615.99
69	1	5	7	1379.60
70	1	2	3	1242.02
71	2	5	2	978.47
72	2	5	7	1348.55

73	3	2	7	941.69
74	2	4	7	1356.30
75	4	4	1	224.77
76	3	1	2	1036.64
77	2	5	6	1480.30
78	2	1	2	964.91
79	2	3	1	1003.66
80	3	4	4	1294.35
81	3	3	3	1290.47
82	2	4	3	1542.30
83	3	1	7	910.69
84	2	3	3	1391.17
85	2	4	2	1315.61
86	1	4	7	1542.36
87	3	1	6	988.20
88	4	5	6	573.54
89	1	3	2	1276.90
90	2	5	3	1265.23
91	1	3	4	2346.48
92	3	2	1	623.92
93	3	3	7	680.11
94	2	1	6	1123.79
95	3	4	5	1240.09
96	1	1	6	881.63
97	4	4	7	422.41
98	2	4	6	1534.55
99	4	1	4	953.32
100	4	1	2	920.38
101	1	4	4	2259.29
102	1	2	1	573.54
103	3	5	7	616.17
104	2	2	1	627.77
105	4	2	6	620.05
106	4	4	2	482.47
107	3	3	6	757.62
108	3	5	1	496.04
109	1	4	6	1767.13
110	4	1	1	707.24
111	2	5	4	1658.56
112	4	1	6	662.67
113	4	2	2	571.60
114	1	2	4	1962.83
115	2	1	3	963.01
116	4	4	6	587.11
117	1	4	5	1898.89
118	4	5	1	56.19
119	1	5	4	2172.09
120	4	1	3	963.01
121	3	4	6	1007.57
122	2	1	1	707.24
123	2	3	6	1730.25
124	3	1	1	517.35
125	1	1	5	955.25
126	4	2	7	511.54
127	3	1	5	1075.39
128	1	3	6	1926.01
129	1	4	3	1530.73
130	3	3	2	1214.90
131	2	2	6	1259.42
132	4	3	5	687.86
133	1	4	1	616.17
134	3	2	3	1530.74
135	3	2	2	1100.58
136	2	4	5	1557.80
137	3	4	7	972.70
138	4	5	4	806.06
139	3	5	6	697.55
140	3	5	4	1201.34

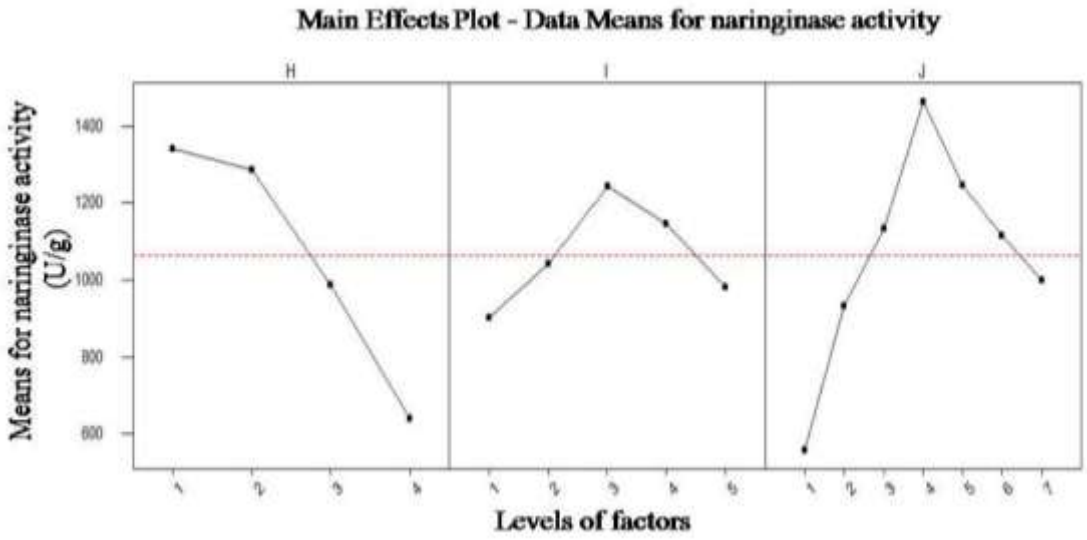

Fig-2: Main effects plot of different conditions on naringinase activity. Here H- temperature, I- pH, and J- fermentation days, where 1,2,3,4 belong to H represent 25ºC, 32ºC, 35ºC, 37ºC and 1,2,3,4,5 belong to I represent pH of 4.0, 4.5, 5.0, 5.5, 6.0 and 1,2,3,4,5,6,7 belong to J represent fermentation days of 3, 4, 5, 6, 7, 8, 9.

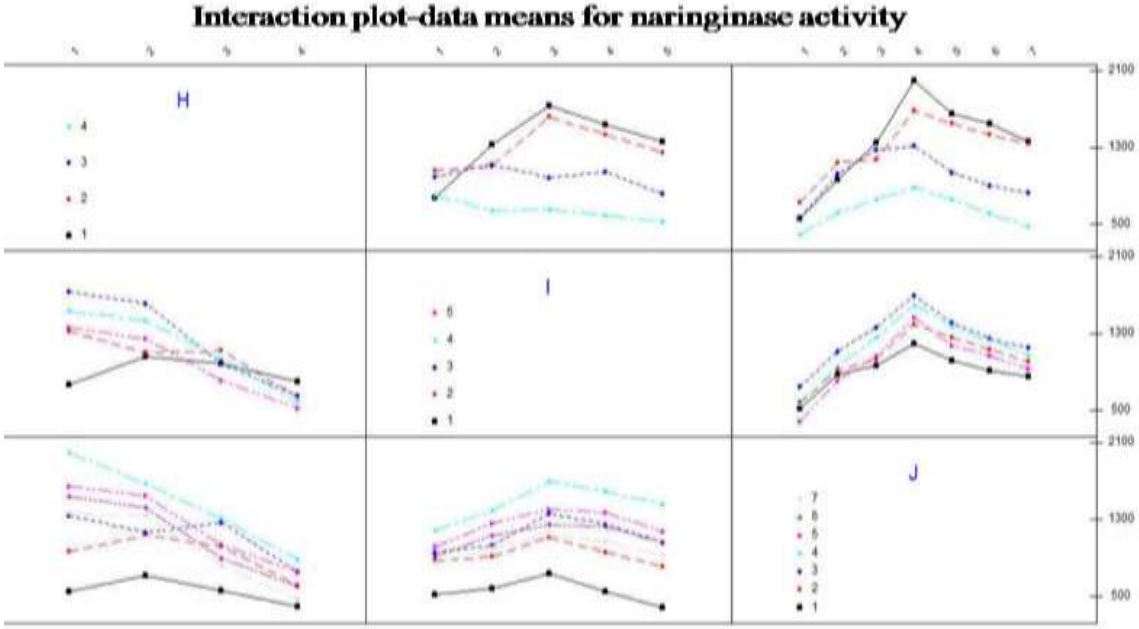

Fig-3: Interaction effects plot of different conditions on naringinase activity. Here H- temperature, I- pH, and J- fermentation days, where 1,2,3,4 belong to H represent 25ºC, 32ºC, 35ºC, 37ºC and 1,2,3,4,5 belong to I represent pH of 4.0, 4.5, 5.0, 5.5, 6.0 and 1,2,3,4,5,6,7 belong to J represent fermentation days of 3, 4, 5, 6, 7, 8, 9.

Table-8: ANOVA table obtained at condition optimization for *Aspergillus flavus* on solid state fermentation.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
H	3	10993815	10993815	3664605	57.65	0.000***
I	4	2025496	2025496	506374	7.97	0.000***
J	6	9597347	9597347	1599558	25.17	0.000***
Error	126	8008784	8008784	63562		
Total	139	30625442				

Here H, I and J namely different levels of temperature, pH and fermentation time.

DISCUSSION

For screening purpose, various C and N sources were evaluated by Placket-Burman design (PB design). PB design mathematically indicates the important factors among several factors investigated. This saves time and provides convincing information for each component in one experiment. Seven factors tested through this design namely naringin, citrus peel, peptone, NH_4NO_3, sucrose, glucose and soya bean extract. The naringin, citrus peel, NH_4NO_3 and sucrose were positively correlated to naringinase activity within tested limits. The increasing concentration of peptone has negative effect on naringinase activity. This agrees with that stated peptone supplemented with ammonium nitrate showed highest enzyme production by *Serratia* Sp [12], sucrose exhibited highest naringinase activity [14], and naringin stimulate enzyme activity [13]. But in another study, peptone proved to have positive effect on naringinase activity through PB design where tested limit (g/L) and microorganism used for production were 7.5-2.5 and *Aspergillus flavus*.

The fungus *Aspergillus flavus* which was isolated from decayed citrus fruits showed highest activity at acidic medium (pH 5.0) at 25ºC. Although marine derived *Aspergillus niger* exhibit highest activity at basal medium [15], *Aspergillus niger* MTCC 1344 showed decreased activity below pH 4.0 [13], and culture conditions optimal for *A. foetidus*, *A. niger* and *A. niger* HPD2 were as follows: pH 5.4, 35°C; pH 5.4, 35°C; pH 5.4, 40°C [16].These phenomena indicated the optimum condition for naringinase activity differs with type of microorganism used for production and where the microorganism gets for production. *Aspergillus flavus* was showed considerable activity at 32ºC also. But increasing the temperature about that showed decreased activity. Similar observation was obtained to *Aspergillus niger* MTCC 1344, where at 37ºC enzyme activity was reduced drastically [19].

The *Aspergillus flavus* produced highest naringinase activity on the 6^{th}day of fermentation. Similar result was reported with another fungi *Aspergillus niger* in solid state fermentation system [17].

CONCLUSION

The solid stage fermentation system using paddy husk as support is a feasible and economical method for the production of naringinase from *Aspergillus flavus* based on the fact that paddy husk is one of the cheap and abundant agro-waste. Apart from other components that positively affect naringinase activity, citrus peel also significantly increases naringinase production. This study concludes that the usage of citrus peel significantly increases naringinase activity of *Aspergillus flavus*. Optimization of culture conditions and the solid fermentation media resulted in 3.74% increase in the naringinase production from *Aspergillus flavus*. Further this method eliminates the issue of citrus peel waste disposal in citrus fruit processing industries.

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