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ORIGINAL ARTICLE

Exploring Waste Citrus Peel as Raw Substrate for Naringinase Production from *Paenibacillus stellifer* RAMCM-44 by Statistical Modelling Using Response Surface Methodology

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ABSTRACT
Naringin is a flavonoid naturally present in citrus fruits contributing bitterness of extracted citrus fruit juices. Naringinase is an enzyme that catalyses naringin hydrolysis which is an environment friendly approach for debittering of citrus juices. The bacterial sources of naringinase have not been studied extensively. In current study, naringinase production has been investigated from Paenibacillus stellifer RAMCM-44 using waste citrus peel powder (CPP) as cost effective raw substrate. Response surface methodology (RSM) has been employed for optimal production of naringinase at shake flask. Five variables optimized were peptone (0.5-1%, w/v), yeast extract (0.4-0.8%, w/v), NaCl (0.5-1%, w/v), citrus peel powder (1-3%, w/v) and KH2PO4 (0.5-1%, w/v). The optimized levels were determined for peptone (0.5%), Yeast extract (0.79%), NaCl (0.53%), citrus peel powder (1.97%) and KH2PO4 (0.5%). Higher naringinase activity (6.47 IU/ml) was obtained with usage of optimal concentrations of media constituents as compared to initial production (4.2 IU/ml). The citrus peel powder was found to act as raw source of inducer (naringin) and has profound effect on naringinase production. The enzyme may be used for de-bittering of citrus juice over chemical processing and waste citrus peel may be used for microbial naringinase production.
Keywords: *naringinase, citrus peel powder, response surface methodology (RSM), Paenibacillus stellifer, citrus peel powder (CPP)*

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INTRODUCTION

Naringin is mainly present in citrus fruits that contribute bitterness to the citrus juices [1,2]. Naringinase is a potent industrial enzyme which catalyses the hydrolysis of bitterness causing flavonoid naringin into naringenin, which in turn is non-bitter and tasteless flavanone. Naringinase mediated naringin hydrolysis is an important development for food industries particularly, for the citrus fruits processing industries. Further, this process is also significant in other food and pharmaceuticals industries due to its ability to reduce bitterness and improvement of the flavour of citrus products, without affecting potential health benefits [3]. Naringin hydrolysis and debittering of citrus fruit juices by naringinase enzyme is one of the promising and environment friendly techniques [4] as compared to chemical processing. Naringinase can be produced by microbes as well as plants [5] but for higher production of naringinase, microbial sources (including fungi and bacteria) have been investigated. Though, majority of investigations on naringinase are based on fungal sources but various bacterial sources have also been suggested promising [6-8]. The

production of naringinase from microbes is influenced by various factors including substrate concentration, inducer and other media constituents used for cultivation of producer microorganism [8-11].

Naringin is the most important inducer substrate for naringinase production. Various reports have investigated pure naringin as well as citrus peel (contains naringin), that serves as a potent inducer for naringinase production [6-8]. Use of citrus peel as a substrate for naringinase production can be cost effective due to its availability and lower cost in comparison to pure substrate (naringin).

Waste citrus peel is generally discarded and considered useless. The use of citrus peel as potent media component may support the concept of waste utilization as environment friendly approach for production of industrial enzymes. In current study, waste citrus peel powder has been investigated as media component and inducer for naringinase production from *Paenibacillus stellifer* RAMCM-44, an isolate of our lab [8,9]. The media components have major role in naringinase production from microbial sources. Different media components exert significant impact on enzyme production at individual as well as in combination. Response surface methodology (RSM) is a well-known statistical tool that is widely used to study the interaction among media components (independent variables) and their relationship with responses [12]. Central composite design (CCD), the most widely used design, has been used to optimize the levels of media components and citrus peel powder for higher production of naringinase in current study.

New efficient bacterial sources of naringinase need to be explored for naringinase production in cost effective manner. In current study, waste citrus peel powder has been used as potent raw substrate for naringinase production from a bacterial source, *Paenibacillus stellifer* RAMCM-44. Majority of earlier studies has been carried on fungal sources. Significant findings of this study may provide useful information on the use of waste citrus peel powder as raw inducer substrate for naringinase production from bacterial sources.

MATERIAL AND METHODS

Materials and chemicals

Bacterial strain *Paenibacillus stellifer* RAMCM-44, a previous isolate of our lab was used as source of naringinase enzyme [8]. The bacterial strain was preserved, maintained and inoculum was developed as described earlier.

Waste peel of citrus fruit commonly known as sweet lemon (*mausambi)* was collected from local market (Mullana-Ambala, Haryana, India). The peel was washed, dried and grinded to prepare fine powder. The powder was filtered through muslin cloth and fine dry powder was stored in air tight containers for further use. The powder was used as raw substrate or inducer in naringinase production media as substitute for pure naringin.

Naringinase assay

Naringinase activity was calculated through naringin hydrolysis [8-9, 13] with some minor modifications as described earlier [8, 10, 14]. Briefly, the fermented broth was centrifuged (7,000 rpm, 10 min, 4°C), and the cell-free broth was used as a crude enzyme to determine enzyme activity. The typical assay mixture consisting 900 µl of naringin (0.05%, w/v) dissolved in sodium acetate buffer (0.1 M; pH 4.5) and 100 µl of crude enzyme (supernatant/cell free broth) was incubated for 1 hour at 50°C. Thereafter, an aliquot of 100 µl from the reaction mixture was taken and added to the 5 mL of diethylene glycol (90%, v/v), followed by the addition of 100 µl NaOH (4 N) solution. The resultant reaction mixture was incubated for 10 minutes at room temperature. The intensity of the developed yellow color was measured at 420 nm in a UV-visible spectrophotometer. A unit of naringinase was defined as the quantity of enzyme that hydrolysed 1 µmol of naringin per minute under the assay's standard conditions.

Experimental design and statistical analysis

Five variables (media components including citrus peel powder) were selected for optimization on the basis of previous studies [8, 9]. Central composite design (CCD) with five variables was employed using response surface methodology (RSM) to reveal the optimal combination of the independent variables (media components) for a common response i.e., naringinase activity. Five variables included: peptone (0.5-1%, w/v), yeast extract (0.4-0.8%, w/v), NaCl (0.5-1%, w/v), citrus peel powder (1-3%, w/v) and KH2PO4 (0.5-1%, w/v), while MgSO4 (0.05%, w/v), MnSO4 (0.001%, w/v) and initial pH of media (6.5±0.2) was kept constant [8, 9] during shake-flask fermentation. Each variable was studied at five coded levels as presented in Table 1. Total 32 experimental runs having different combinations of media components were performed (Table 2). The culture conditions for shake-flask fermentation were same as described earlier [8]. Results of all experimental runs were statistically analysed with Design-Expert (Stat-Ease Inc., Minneapolis, USA). The response obtained in terms of naringinase activity was analyzed

using analysis of variance (ANOVA). Further, the statistical significance in respect of regression coefficients and second-order model equation was also determined. The optimal combination of variables (media components) was obtained for higher production of naringinase.

Table 1: Experimental Range and Levels of Independent Variables in Terms of Coded and Actual Factors

Factors (%, w/v)	Symbols	Actual Levels of Coded Factors				
		Min.	-1	0	+1	Max.
Peptone	A	0.25	0. 50	0.75	1.0	1.25
Yeast Extract	B	0.20	0.40	0.60	0.80	1.00
NaCl	C	0.25	0. 50	0.75	1.0	1.25
CPP*	D	0.0	1.00	2.00	3.00	4.00
KH2PO4	E	0.25	0. 50	0.75	1.0	1.25

*Citrus Peel Powder (CPP)

RESULTS AND DISCUSSION

Waste citrus peel was used for optimal production of industrial important naringinase enzyme from Paenibacillus stellifer RAMCM-44 has been investigated with help of RSM. The central composite design (CCD) was used and a total of 32 experiments with various appropriate combinations of peptone, NH4NO3, NaCl, citrus peel powder and KH2PO4 were conducted (Table 1). The designed experiments and results of naringinase production have been presented in Table 2. The obtained results (experimental) of CCRD were fixed with a second order polynomial equation (as mentioned below). The values of regression coefficients were also calculated and the fitted equations (in terms of coded values, without modification) for naringinase production (X) are given below not considering the significance of the coefficients:

X = +5.27 + 0.1821 * A + 0.0663 * B - 0.0687 * C + 0.1321 * D + 0.3701 * E - 0.0844 * A * B -0.0181 * A * C + 0.4694* A * D – 0.1694 * A * E – 0.2519 * B * C + 0.1231* B * D + 0.3519 * B * E - 0.1381 * C * D + 0.0356 * C * E + 0.0281 * D * E + 0.0945* A2 - 0.0355 * B2 + 0.1057 * C2 - 0.6780 * D2 + 0.0906* E2

(A is peptone concentration, B: NH4NO3 concentration, C: NaCl concentration, D: citrus peel powder concentration and E: KH2PO4 concentration). The statistical significance of above equation was checked through "analysis of variance (ANOVA)" and summarized in Table 3. Further, the significance of model is revealed by very low probability value of naringinase production (Table 3). P values further suggest the significance of each coefficient, which also may be useful in indicating the pattern of interaction among the coefficients. Smaller the P value, more significant is the corresponding coefficient [15]. The R2 value of 0.9972 was obtained for naringinase activity. The Predicted R^2 of 0.9064 is in reasonable agreement with the Adjusted R^2 of 0.9921. The coefficient estimates of equation (1) presented in Table 4. Model F-value of 194.56 was found for naringinase production. The lack of fit "sum of squares" values for naringinase activity has been shown in Table 3.

Table 2: Central Composite Design Matrix for the Experimental Design for Naringinase Production and Biomass Yield

Std	Run	Factor 1 A: Peptone (%; w/v)	Factor 2 B: Yeast Extract (%; w/v)	Factor 3 C: NaCl (%; w/v)	Factor 4 D: Citrus Peel Powder (%; w/v)	Factor 5 E: KH2PO4 (%; w/v)	Response: Naringinase Activity (IU/ml)
27	1	0.75	0.6	0.75	2.0	0.75	5.28
19	2	0.75	0.2	0.75	2.0	0.75	5.02
31	3	0.75	0.6	0.75	2.0	0.75	5.28
26	4	0.75	0.6	0.75	2.0	0.5	5.05
21	5	0.75	0.6	0.25	2.0	0.75	5.75
22	6	0.75	0.6	1.25	2.0	0.75	5.57
3	7	0.5	0.8	0.5	1.0	0.5	4.42
25	8	0.75	0.6	0.75	2.0	0.25	4.81
12	9	1.0	0.8	0.5	3.0	0.5	5.72
16	10	1.0	0.8	1.0	3.0	1.0	5.89
10	11	1.0	0.4	0.5	3.0	1.0	5.35
29	12	0.75	0.6	0.75	2.0	0.75	5.28

5	13	0.5	0.4	1.0	1.0	0.5	5.12
32	14	0.75	0.6	0.75	2.0	0.75	5.28
14	15	1.0	0.4	1.0	3.0	0.5	5.64
11	16	0.5	0.8	0.5	3.0	1.0	5.93
2	17	1.0	0.4	0.5	1.0	0.5	4.54
13	18	0.5	0.4	1.0	3.0	1.0	4.32
8	19	1.0	0.8	1.0	1.0	0.5	3.59
1	20	0.5	0.4	0.5	1.0	1.0	4.77
24	21	0.75	0.6	0.75	4.0	0.75	2.86
9	22	0.5	0.4	0.5	3.0	0.5	3.8
30	23	0.75	0.6	0.75	2.0	0.75	5.28
4	24	1.0	0.8	0.5	1.0	1.0	5.05
17	25	0.25	0.6	0.75	2.0	0.75	5.32
7	26	0.5	0.8	1.0	1.0	1.0	5.78
18	27	1.25	0.6	0.75	2.0	0.75	5.91
15	28	0.5	0.8	1.0	3.0	0.5	3.2
23	29	0.75	0.6	0.75	0.0	0.75	1.19
20	30	0.75	1.0	0.75	2.0	0.75	5.17
28	31	0.75	0.6	0.75	2.0	0.75	5.26
6	32	1.0	0.4	1.0	1.0	1.0	4.75

Naringinase production prediction by the model along with the experimental observed values have been shown in Table 5, which shows an outstanding agreement between the predicted and experimental values. The optimal concentrations (%) obtained by differentiation of the quadratic model for achieving maximum naringinase production and biomass yield were A = 0.50, B = 0.79, C = 0.52, D = 1.97 and E = 0.5. The accuracy of the model was assessed by experiment in triplicates using these derived medium components. The experiment leads to an average naringinase production of 6.47 (IU/ml). The better agreement between the predicted (6.21 IU/ml) and experimental value (6.47 IU/ml) revealed the validity of the model along with the existence of optimal point.

Table 3: Analysis of Variance (ANOVA) for Response Surface Quadratic Model Obtained from Experimental Design

Source	**Naringinase Activity**		
	Sum of Squares	**DF**	**Prob.>F**
Model	25.63	20	0.0001
A-Peptone	0.7957	1	0.0001
B-Yeast Extract	0.1053	1	0.0021
C-NaCl	0.1134	1	0.0016
D-Citrus Peel Powder	0.4187	1	0.0001
E-KH2PO4	2.36	1	0.0001
AB	0.1139	1	0.0016
AC	0.0053	1	0.3908
AD	3.53	1	0.0001
AE	0.4590	1	0.0001
BC	1.02	1	0.0001
BD	0.2426	1	0.0001
BE	1.98	1	0.0001
CD	0.3053	1	0.0001
CE	0.0203	1	0.1069
DE	0.0127	1	0.1931
A^2	0.2593	1	0.0001
B^2	0.0367	1	0.0378
C^2	0.3248	1	0.0001
D^2	13.36	1	0.0001
E^2	0.1367	1	0.0008
Residual	0.0724	11	
Lack of Fit	0.0721	6	0.0001
Pure Error	0.0003	5	
Core Total	25.70	31	

In current study, citrus peel powder (CPP) seems to be an important and potent raw substrate for naringinase production. The CPP served as raw source of naringin as well as carbon source. The media components efficiently interacted with each other which results in significant effect on naringinase production from *Paenibacillus stellifer* RAMCM-44 as presented in Table 2. As revealed in Table 2 (run number 1, 5, 9, 10, 16 and 27) with increase in concentration of CPP from 1% (w/v) to 3% (w/v), the naringinase production also increased. It has been observed that increase in concentration of CPP up to 3% (w/v) supports higher production of naringinase but, CPP more than 3% (w/v), did not significantly affected the production. At higher concentration, 4% (w/v), the naringinase production was suppressed which may be due to inhibitory effect of various compounds present in CPP at higher concentrations. Higher naringinase activity (5.93 IU/ml) was observed in experimental run number 16 (Table 2) with concentrations of peptone, NH4NO3, NaCl, citrus peel powder and KH2PO4 as 0.50 (%; w/v), 0.80 (%; w/v), 0.50(%; w/v), 3.0 (%; w/v) and 1.0 (%; w/v), respectively.

Table 4: Regression Coefficients and Significance of Quadratic Model for Naringinase Activity

Model	Naringinase Activity		
	Coefficient Estimate	Standard Error	F-value
Intercept	5.27	0.0311	194.56
A-Peptone	0.1821	0.0166	120.82
B-Yeast Extract	0.0663	0.0166	15.99
C-NaCl	-0.0687	0.0166	17.22
D- Citrus Peel Powder	0.1321	0.0166	63.58
E-KH2PO4	0.3701	0.0195	358.38
AB	-0.0844	0.0203	17.30
AC	-0.0181	0.0203	0.7981
AD	0.4694	0.0203	535.24
AE	-0.1694	0.0203	69.70
BC	-0.2519	0.0203	154.13
BD	0.1231	0.0203	36.83
BE	0.3519	0.0203	300.81
CD	-0.1381	0.0203	46.35
CE	0.0356	0.0203	3.08
DE	0.0281	0.0203	1.92
A^2	0.0945	0.0151	39.37
B^2	-0.0355	0.0151	5.57
C^2	0.1057	0.0151	49.31
D^2	-0.6780	0.0151	2028.51
E^2	0.0906	0.0199	20.76

Along with CPP, peptone also exert positive impact on naringinase production (run 27, Table 2). At a higher concentration (1.25 %; w/v), peptone along with CPP (3%; w/v) resulted in better production of naringinase as compared to lower concentrations (Table 2). Similarly, yeast extract, NaCl and KH2PO4 has significant interactions that positively affect naringinase production (Table 2). The interaction among various factors have been shown in Figure 1.

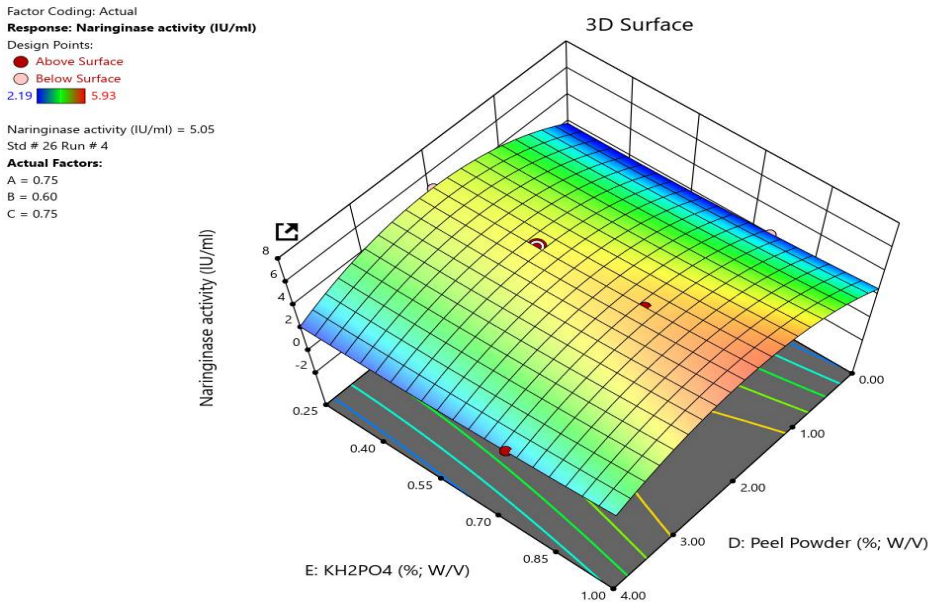
The results support earlier findings of naringinase production from various microbial sources including bacteria. Citrus peel powder (CPP) from fungal as well as bacterial sources has been earlier investigated by various researchers for naringinase production [6]. However, limited literature is available in bacterial production of naringinase using CPP. Further, peptone, yeast extract, NaCl and KH2PO4, MgSO4, MgSO4, MnSO4 and citrus peel powder (CPP) has already been reported as part of production media in different reports on microbial naringinase production. The role of citrus peel powder as an inducer for naringinase has been investigated and found to be potent raw substrate for production of naringinase from efficient microbial strains. The interaction among various media components has been revealed and the results showed positive impact of these components on enzyme production.

Table 5: Predicted Values vs Experimental Values for Maximum Naringinase Activity

Factors	Concentration (%; w/v)	Naringinase Activity (IU/ml)	
		Predicted Value	Experimental Value
Peptone (A)	0.50	6.21	6.47
Yeast Extract (B)	0.79		
NaCl (C)	0.52		
Citrus Peel Powder (D)	1.97		

KH2PO4 (E)	0.50		

Earlier, media components for production of naringinase from *Paenibacillus stellifer* RAMCM-44 were optimized by *one variable at a time* and as function of concentration of media components. However, the interaction between different components of media and their impact on naringinase production cannot be determined properly by traditional method (*one variable at a time*).



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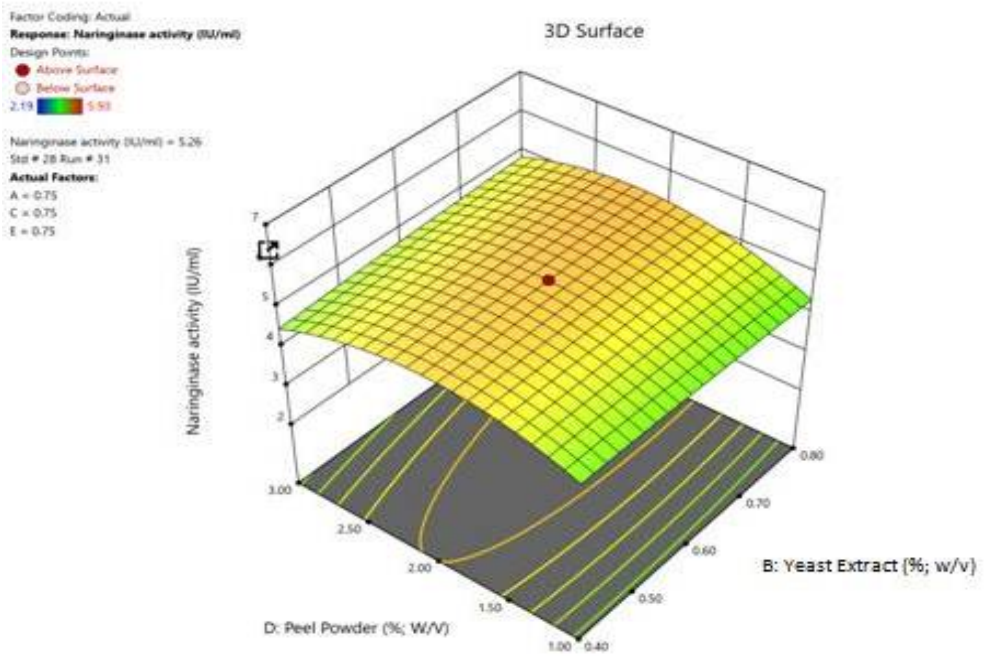

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Figure 1: Response surfaces showing the effect of varying concentrations of citrus peel powder (CPP) and KH2PO4 (A); citrus peel powder (CPP) and yeast extract (B) on naringinase production. The interaction among two variables has significant effect on naringinase production.

Application of RSM has allowed inclusion of interactive effects in considering the various media components for higher naringinase production. Higher production of naringinase revealed application of statistical as well as mathematical models to obtain desired response from the biological processes.

Though, some reports are available on naringinase production using citrus peel powder (CPP) but, production of this potent enzyme from bacterial source, *Paenibacillus stellifer* RAMCM-44 using waste citrus peel powder with help of RSM has been reported first time. Citrus peel powder can be used as an inducer as well as potent carbon source.

Previous studies on naringinase production are mostly from fungal sources. Very few bacterial sources have been explored for naringinase production. In current study, bacterial source has been investigated for optimal production of naringinase using citrus peel powder. Previously, different raw substrates have been reported for naringinase production using different microbial strains. In particular, citrus waste (peel) has been used in majority of studies due to presence of naringin in citrus peel. In 2011, researchers used *S. xylosus* MAK2 for naringinase production using raw citrus peel powder as source of naringin [16]. Authors also optimized the carbon source, nitrogen source and metal ions for higher production of naringinase. The higher naringinase production yield of 6.1 IU/ml was obtained with 2% (w/v) of citrus peel, and further increase in concentration of inducer lead to decreased production of enzyme.

The results of current study also revealed almost similar pattern (Table 2). The production of enzyme naringinase was increased up to concentration of 3% (w/v) and beyond this concentration; decrease in naringinase production was obtained (Table 2). In a similar study, Borkar et al. [17] have reported production from *Aspergillus niger* van Tieghem MTCC 2425. The production of enzyme was carried using citrus wastes. It was observed that that pH, temperature of incubation, and inducer concentration are most important factors affecting production of naringinase. A rotatable central composite design (CCD) was employed to obtain optimal levels of these factors [17]. Mendoza Cal et al. [18] have reported naringinase production using twelve filamentous fungi in solid substrate fermentation. Authors used orange as well as grapefruit rind in form of substrate containing naringin. The fungal strains included strains of *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. The fungal naringinase was able to hydrolyze the naringin significantly. The highest naringinase activity was obtained from *Aspergillus foetidus* (2.58 U/ml). The production was carried using citrus peel and mineral salt solution containing KH2PO4, K2HPO4, NH4Cl, MgSO4.7H2O, and FeCl3. Nutritional requirements of *Aspergillus oryzae* JMU316 for production of naringinase has been reported by Chen et al. [19]. Naringin was reportedly found to be the most efficient and effective carbon source followed by pomelo pericarp. Among nitrogen sources, peptone found to be most suitable component. Along with these major components, the media was supplemented with minerals including CaCl2, NaCl, MgSO4·7H2O, and K2HPO4. Xia et al. [20] used *Aspergillus tubingensis* UA13 for production studies of naringinase. Authors optimized the production of enzyme at shake flask level and further at bioreactor level. Among naringin and pomelo peel powder, naringin was found to support higher naringinase production as compared to pomelo peel powder. It was also found that additional carbon sources did not allow further increase in naringinase level. Oliveira et al. [21] have studied production of naringinase from *Aspergillus niger strain* 426. Response surface methodology (RSM) was used for optimal production of naringinase using solid substrate fermentation (SSF). Three substrates namely, grapefruit peel, rice bran, and wheat bran were assessed for their potential to be used as substrate. The substrates were supplemented with mixture of naringin, rutin, and hesperidin. Carbon source, nitrogen source and inducer play an important role in microbial production of naringinase. Similarly, Bodakowska-Boczniewicz and Garncarek [22] synthesized naringinase from *Aspergillus niger* using naringin, along with powdered albedo, flavedo, and red grapefruit segment membranes as stimulators. The carbon sources used was rhamnose while yeast extract and sodium nitrate were used as nitrogen sources. Optimal production was obtained with NaNO3, yeast extract, KH2PO4, red grapefruit albedo, naringin, and rhamnose. Balaraman et al. [23] have reported production of naringinase from *Bacillus amyloliquefaciens.* Recently, a bacterial source, *Bacillus subtilis* strain BSnari, an isolate from the Red Sea has been reported for naringinase production with activity of 7.09 U/ml [24]. The media was majorly included sucrose (1.5%), citrus peel powder (0.6%) and soybean meal (1%) with pH of 7. The growth was carried at 37ºC. Some other studies have also discussed the use of citrus peel powder and related raw substrates for production of microbial naringinase through fermentations [25-28]. Our results corroborate with earlier findings on production of bacterial as well as fungal naringinase using raw substrates chiefly, citrus fruit wastes. Citrus peel powder seems to be potent and promising raw substrate that may provide sufficient naringin as inducer for naringinase production by specific microbial strains [6-8]. The interaction among various media components has been revealed and the results showed significant impact of these components on enzyme production.

The microbes are routinely cultivated for various metabolites and enzyme production for industrial purposes. Different media components have been shown to exert great impact on growth and product formation through fermentations [29]. In current scenario, use of agro-industrial waste as substrate for industrial fermentations has gained importance. Waste utilization solves the problem related to waste

management, environment sustainability and value addition to wastes. Current study supports the usage of waste citrus peel that is generated in a large amount and generally goes waste. Production of industrial enzyme like naringinase may help to the use of waste citrus peel along with the biological de-bittering of juice using naringinase. Further investigations on *Paenibacillus stellifer* RAMCM-44 may add to the existing knowledge of naringinase production from bacteria which otherwise is very limited as compared to fungal sources.

CONCLUSION

Media components play important role in production of microbial enzymes of industrial interest. Naringinase production from *Paenibacillus stellifer* RAMCM-44 has been studied by optimization of media components with help of RSM. The optimal levels of peptone, yeast extract, NaCl, citrus peel powder (CPP) and KH2PO4 has been determined and validated. Citrus peel powder was used as inducer for naringinase production. Production of naringinase in response to naringin present in CPP reveals that CPP has potential to be used as cost effective raw substrate. Use of agricultural wastes as raw substrates has gained interest of researchers due to low cost, easy availability, utilization of waste and other benefits. Production of naringinase by *Paenibacillus stellifer* RAMCM-44 using CPP as substrate can be stimulated with change in concentration of media constituents. The optimal combination of media components may be used for further studies on scale up and also for optimization of culture conditions. The findings may provide useful insights into production of bacterial naringinase that has been reported to lesser extent as compared to fungal sources. The study may be used for scale up studies and application of enzyme for hydrolysis of naringin in citrus juices.

Conflicts of interests

The authors declare no any conflict of interest.

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Author Contributions

RN conducted experiments and collected data; NS helped in writing manuscript, AK (Amit Kumar) and SK, SKU helped in data analysis, MS and AK (Ashwani Kumar) helped in improving manuscript language and provided suggestions, Mukesh Yadav (MY) planned the research, analysed data and finalized the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this published article.

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Not Applicable

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