Research Article

Molecular Characterization of a New Bacterial Source of Naringinase as *Paenibacillus stellifer* RAMCM-44 and Fermentative Production of Naringinase at Shake-Flask Level

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ABSTRACT

Aim/Background: Naringin is an important flavonoid present naturally in the citrus fruits. It contributes bitterness to the citrus fruit juices. Biologically, the naringin mediated bitterness can be reduced by naringinase mediated hydrolysis of naringin. The naringinase is an enzyme complex that hydrolyses naringin and produces tasteless product naringenin. **Materials and Methods:** In current study, naringinase producing microbial strains (bacteria) were isolated from soil adjacent to the citrus plants roots (rhizosphere) and screened for their naringinase producing potential. The higher naringinase producing bacterial strain RAMCM-44 was characterized by 16s rRNA gene sequencing, phylogenetic analysis. The fermentative production of naringinase was studied at shake-flask level and optimized by *one-variable-at-a-time* method. **Results and Conclusion:** Naringinase producing bacterial strain was isolated and characterized to be *Paenibacillus stellifer*. The bacterial strain was able to produce naringinase in response to both naringin as well as citrus peel powder as raw substrate. The Paenibacillus sp. has not been explored widely and remained to be studied extensively. This is first report on production of naringinase from *Paenibacillus stellifer* and adds to existing knowledge about *Paenibacillus stellifer*.

Keywords: Naringinase, *Paenibacillus stellifer*, 16S rRNA gene sequence, Fermentation, Media Optimization.

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INTRODUCTION

Naringin is a well known natural flavonoid generally found in citrus fruits. Naringin contributes to the bitter taste of the citrus fruit juices.[1,2] The gradual inception of bittering in citrus fruits juice is very important drawback for their use and storage. Also, this bitterness is undesirable and therefore exists as a major concern for the related food industries.[3] Accordingly, level of naringin should be reduced or removed from the processed products.[4] The naringin mediated bitterness can be reduced by decreasing the naringin content through chemical methods, but the chemical method has several drawbacks and also results in inferior quality of the extracted fruit juice.[5] Naringinase enzyme has the potential to hydrolyze the bitter naringin in to a tasteless product naringenin and therefore, have ability to lessen the citrus juices bitterness.[6,7] The microbial naringinase has potential to replace the chemical methods to reduce the bitterness.

Naringinase consists of two different catalytic activities i.e. α-L-rhamnosidase along with β-D-glucosidase activity that works in a sequential manner. Naringinase hydrolyze the naringin into naringenin in two steps. In first step, naringin is hydrolyzed to rhamnose and

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prunin (glucoside) due to α-L-rhamnosidase activity of naringinase, while in second step, prunin hydrolysis lead to production of naringenin and glucose due to β-D-glucosidase activity of naringinase.[2,8-10] Out of these two catalytic activities of naringinase, the rhamnosidase activity is considered necessary to eliminate bitterness.[11] Naringinase has the potential to be used at industrial level for de-bittering of citrus juices. Several studies have been performed on microbial naringinase for hydrolysis of naringin in citrus juices and results have been found promising.[12] But most of the studies focused on fungal sources of naringinase and bacterial sources need to be explored more extensively due to their some obvious advantages over fungal counterparts.

Bacteria have been considered important source of various industrial enzymes and metabolites.[13-16] A large number of enzymes have been reported from various bacterial sources for potent industrial applications.[17-19] In this article, a naringinase producing bacterial strain, *Paenibacillus stellifer* RAMCM-44, has been isolated from rhizosphere of citrus roots. The isolated strain was characterized by 16s rRNA gene sequencing and phylogenetic analysis. The isolated bacterial strain was identified as *Paenibacillus stellifer* and named as *Paenibacillus stellifer* RAMCM-44. The bacterial strain was found to produce naringinase in media containing pure naringin as well as citrus peel powder Optimization of media constituents was performed at shake flask level using *one-variable-at-a-time* method for naringinase production by *Paenibacillus stellifer* RAMCM-44. Further, agricultural waste material (citrus peel powder) has been assessed for potential to be used as raw substrate for production of naringinase. Further studies are required on characterization and application of the microbial naringinase produced from naringinase. This is first report on *Paenibacillus stellifer* as source of naringinase.

MATERIALS AND METHODS

Collection of soil samples

The samples of soil were collected from various locations/ fields of Haryana (India) having citrus plantations. The samples were taken from the immediate surroundings of roots (citrus plants) and carried to laboratory in air tight pre-sterilized plastic bottles. Samples were stored in their natural state at 4ºC, until further use.[20,21]

Isolation and screening of naringinase producing bacterial strains

The naringinase producing bacteria were isolated from soil samples.[22-25] Serial dilution approach was used for isolation of naringinase producing microbial strains. Spreading of appropriately diluted soil samples was done on the nutrient agar media (peptone, 0.5% w/v; yeast extract, 0.3% w/v; NaCl, 0.5% w/v; Agar, 1.5% w/v; pH 6.5) containing citrus peel powder (1.0% w/v). The plates were incubated at 37ºC for 24 hr. Fast growing, larger colonies were selected and cultured on nutrient agar media (peptone, 0.5% w/v; yeast extract, 0.3% w/v; NaCl, 0.5% w/v; Agar, 1.5% w/v; pH 6.5) containing naringin (0.05%; w/v). Based on faster growth and larger size, the microbial colonies were selected and preserved for screening on the basis of naringinase activity. The isolated strains were observed using microscope and bacterial strains were picked and stored for further use.

The isolated microbial strains were grown separately in nutrient broth (same as isolation media except agar, pH 6.5) supplemented with naringin (0.05%; w/v) for 24 hr at 37ºC with continuous agitation of 150 rpm in an orbital shaker. After 24 hr of incubation, fermented broth was harvested and centrifuged (7000 rpm; 4ºC; 10 min) to separate the cells from broth. The supernatant (cell free broth) was taken as crude enzyme and used to measure the naringinase activity.[26-28] Depending on the naringinase activity, microbial strain RAMCM-44 was selected for further investigations and naringinase production.

16S ribosomal RNA gene sequence of naringinase producing bacterial strain RAMCM-44 and phylogenetic analysis

Based upon the microscopic observations, the microbial strain RAMCM-44 was confirmed to be bacteria which was further characterized by 16s RNA gene sequencing followed by phylogenetic analysis. The 16S rRNA gene was amplified and sequenced at geneOmbio Technologies Pvt. Ltd., Pune, India. The homology of the obtained 16S ribosomal RNA gene sequence (partial sequence) was determined using BLAST program of NCBI, USA (https://blast.ncbi.nlm.nih.gov) against the NCBI nucleotide database.[29] The sequence data obtained for the 16S rRNA gene was aligned against the reference nucleotide sequences of the genera retrieved from the NCBI, GenBank using ClustalW program.and analyzed to find the closest homolog for the bacterial strain (RAMCM-44).[30] The phylohgenetic tree was constructed using BLAST pairwise alignments with neighbor-joining method with the help of online service provided by NCBI, USA (https://www.ncbi.nlm.nih.gov).[31] The bacterial isolate was identified to be *Paenibacillus stellifer* RAMCM-44.

Production of naringinase by *Paenibacillus stellifer* RAMCM-44 as function of media components

The media used for isolation and screening was taken as basal media (peptone, 0.3% w/v; yeast extract, 0.5% w/v; NaCl, 0.5% w/v; naringin, 0.05% w/v) and additional media components were assessed to support higher production of naringinase. Additional media components were selected on the basis of earlier reports on naringinase production.[24,25,27,28, 32]. Media components including KH_2PO_4 (0.3%, w/v), K_2HPO_4 (0.3%, w/v) and $MgSO_4$ (0.05%, w/v), $MnSO_4$ (0.001%, w/v) were assessed for their effect on naringinase production. After preliminary studies, concentration of major media components including yeast extract (0.1%-0.5%, w/v), peptone (0.3%-0.7%, w/v), NaCl (0.3%-0.7%, w/v), naringin (0.05-0.25%; w/v), KH_2PO_4 (0.3%-0.7%, w/v) and $MgSO_4$ (0.05%-0.25%, w/v) were optimized by using *one-variable-at-a-time* approach in order to obtain the significant value of media components.

Citrus peel powder (CPP) as raw substrate for naringinase production

Waste peel of four citrus fruits (lemon, sweet lemon or mosambi, orange and kinnow; 1.0%, w/v) was studied as potent raw substrate for naringinase production from *Paenibacillus stellifer* RAMCM-44. Clean unspoiled peel was dried, grinded, filtered and used as raw substrate. All other media constituents were used at their optimal concentrations.

Assay for enzyme (naringinase) activity

Naringinase activity was measured on the basis of naringin hydrolysis as described by Davis[26] with minor modifications.[27,28] Briefly, the fermented broth was subjected to centrifugation (7,000 rpm for 10 min at 4°C) and the cell free broth or supernatant was used as crude enzyme to determine enzyme activity. The assay mixture, consisted of 900 µL naringin (0.05%, w/v) prepared in sodium acetate buffer (0.1 M; pH 4.5) and 100 µL crude enzyme (supernatant; cell free broth). The mixture was incubated at 50°C for 1 hour. Thereafter, an 100 µL aliquot from the reaction mixture was taken and mixed with 5 mL of diethylene glycol (90%, v/v) followed by addition of NaOH; 100 µL (4 N) solution. After this, the reaction was incubated for 10 min at room temperature. The intensity of the developed yellow color was recorded at 420 nm in a UV-visible spectrophotometer. One unit of naringinase was defined as the amount of enzyme hydrolyzing 1 µmol of naringin per minute under the standard conditions of the assay.

RESULTS

Isolation and screening of naringinase producing microbial strains

Appropriate dilutions of soil samples were spread on citrus peel powder containing agar media and total 40 microbial colonies were isolated. The colonies were cultured on nutrient agar media containing naringin. Depending on growth and size of colony, total fifteen isolates were selected, confirmed to be bacteria by microscopic observation and preserved for screening.

Fifteen bacterial isolates were screened for the naringinase activity. The isolate RAMCM-44 (Rachna, Anushka, Mukesh, Citrus Soil, Mullana, isolate 44) showed higher naringinase production (2.8±0.06 IU/ml) followed by isolate RMCSJ-28 (2.5 IU/mL), KMSM-18 (2.2 IU/mL) and RMCM-11 (1.64 IU/mL) (Figure 1). The isolate RAMCM-44 was selected for further studies on naringinase production and also for identification by 16S rRNA gene sequencing analysis. Though, both fungal and bacterial sources have been explored for naringinase production, but, majority of the research work on naringinase production and related aspects has been carried out using fungal strains. In current study, isolation of naringinase producing bacterial strains was the main focus. Naringinase activity was used as the quantitative approach for screening of the isolates. The bacterial isolate RAMCM-44 was identified and characterized by performing 16S rRNA gene sequencing and phylogenetic analysis.

Isolation of different naringinase producing microbial strains has been reported earlier by various researchers and some strains have been isolated from soil,[32] citrus peel spoiled in air or soil[33] and other samples.[34]

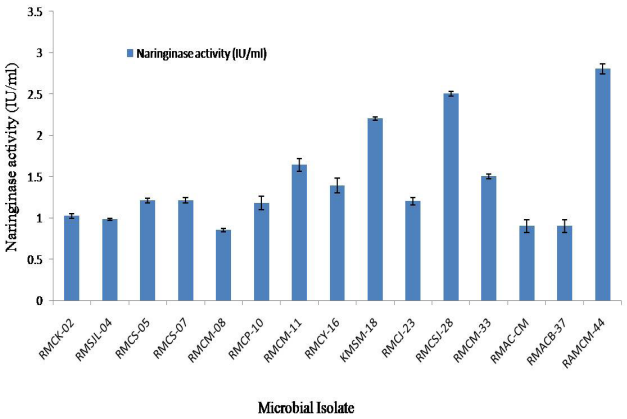

Figure 1: Naringinase activity of different bacterial isolates screened for naringinase production.

16S ribosomal RNA gene sequence of bacterial strain RAMCM-44 and phylogenetic analysis

The comparative analysis and homology of the 16S rRNA gene sequence (533 bp) of the isolate RAMCM-44 using nucleotide blast-NCBI revealed highest similarity with the *Paenibacillus stellifer*. Therefore, the bacterial strain RAMCM-44 was identified as *Paenibacillus stellifer* and named as *Paenibacillus stellifer* RAMCM-44. The taxonomical information of the bacteria was found to be as follows: Bacteria; Bacillota; Bacilli; Bacillales; Paenibacillaceae; Paenibacillus. The phylohgenetic tree was constructed (Figure 2) using BLAST pairwise alignments with neighbor-joining method of Saitou and Nei[31] with the help of online platform provided by NCBI, USA (https://www.ncbi.nlm.nih.gov). This is the first report revealing naringinase producing potential of *Paenibacillus stellifer*. Majority of research have focused naringinase production from fungal sources. Though, some promising bacterial sources of naringinase have also been reported and naringinase from these bacterial sources have been investigated for various bioprocess aspects. The bacterial strains have been studied for production of naringinase as well as rhamnosidase.

Isolation of different bacterial strains belonging to genus *Paenibacillus* have been reported from various environments and majority of them has been reported from soil. In soil, these strains have been found in association with plant roots.[35] Few species of *Paenibacillus* have been earlier recorded to produce useful antimicrobial compounds[35] and also enzymes having potential applications in various fields. The bacteria *Paenibacillus stellifer* has also been investigated for promotion of plant growth and for alleviating salt stress.[36] The *Paenibacillus stellifer* has also been isolated form paperboard and reported to produce cyclodextrin.[37] In current study, the *Paenibacillus stellifer* has been isolated from soil surrounding the citrus plant roots and have been found promising for production of naringinase.

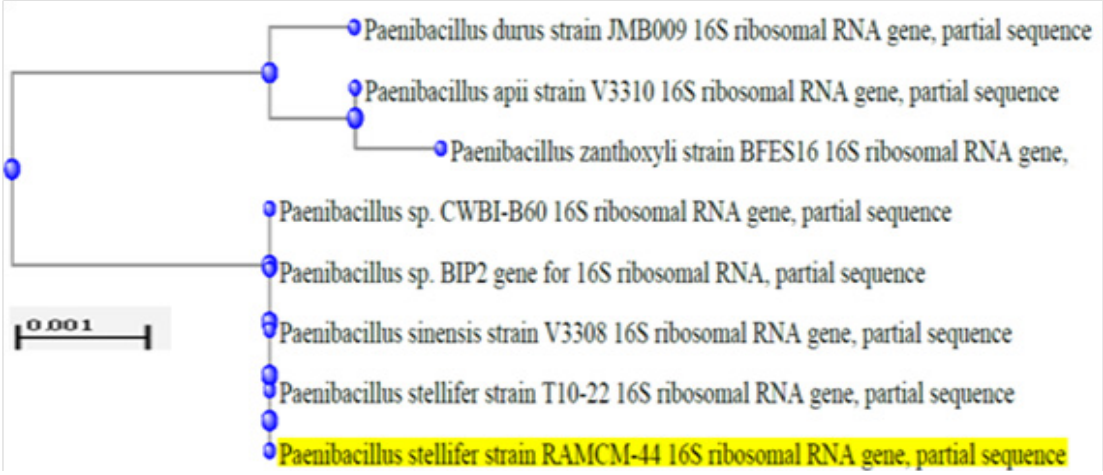

Figure 2: Phylogenetic tree showing relationship of the 16S rDNA sequence of the *Paenibacillus stellifer* RAMCM-44 with close homologs of the *Paenibacillus* sp. The phylohgenetic tree was constructed using BLAST pairwise alignments using the online platform provided by NCBI, USA.

Submission of nucleotide sequence at GenBank, NCBI (USA) and accession number

The 16S ribosomal RNA gene sequence (partial) was submitted to GenBank (NCBI, USA) data base and has been assigned accession number OR096070.

Production of naringinase by *Paenibacillus stellifer* RAMCM-44 as function of media components

Depending on the preliminary studies, both KH_2PO_4 (0.5%, w/v) and $MgSO_4$ (0.05%, w/v) were included in final production media while, K_2HPO_4, was found to be non-significant and therefore, not included in production media. Optimization of the concentration of following media components including yeast extract (0.1%-0.5%, w/v), peptone (0.3%-0.7%, w/v), NaCl (0.3%-0.7%, w/v), naringin (0.05-0.25%; w/v), KH_2PO_4 (0.3%-0.7%, w/v) and $MgSO_4$ (0.05%-0.25%, w/v) was done using *one-variable-at-a-time* approach.[21,38,39] in order to obtain the significant value of media components. The concentration of $MnSO_4$ (0.001%, w/v) was kept constant in all experiments during media optimization. Yeast extract resulted in higher production of naringinase at 0.4% (w/v). Further increase in concentration did not significantly increase the naringinase production (Figure 3). The optimal level for peptone and NaCl was found to be 0.5% (w/v). Naringin lead to higher production at 0.15% (w/v). At higher concentrations of naringin, no significant increase was revealed (Figure 3). The

optimal concentration of KH_2PO_4 was found to be 0.5% (w/v), while $MgSO_4$ showed higher activity at 0.05% (w/v). Further increase in its concentration did not give significant improvement in naringinase production (Figure 3). The optimized concentrations of media components obtained were as: yeast extract, 0.4% (w/v); peptone, 0.5% (w/v); NaCl, 0.5% (w/v); naringin, 0.15% (w/v); KH_2PO_4, 0.5% (w/v) and $MgSO_4$, 0.05% (w/v). $MnSO_4$ was incorporated at constant concentration (0.001%, w/v). Naringinase production as function of media components have been shown in Figure 3. The optimizations lead to higher naringinase production (5.29 IU/mL) as compared to initial production (2.8 IU/mL). *One-variable-at-a-time* is still widely used methodology to optimize various media components and to study the production of microbial products as function of media components. Earlier, various authors have reported optimization of media components for higher production of naringinase using bacterial as well as fungal sources.[5,40] Production of naringinase from bacterial sources still requires extensive studies and exploration of microbial sources for higher production of naringinase at commercial level. Various media components have been shown to affect the naringinase production using different microbial strains including both bacteria and fungal strains. Earlier studies on production of naringinase from *Micrococcus* sp. have reported use of similar media components including KH_2PO_4, $MnSO_4$ and $MgSO_4$ while naringin was used as major inducer substrate.[32] Similarly, KH_2PO_4, NaCl, $MgSO_4$ and yeast extract have also been used for the production of naringinase from *Serratia* Sp. authors also used naringin as potent inducer for naringinase production.[28]

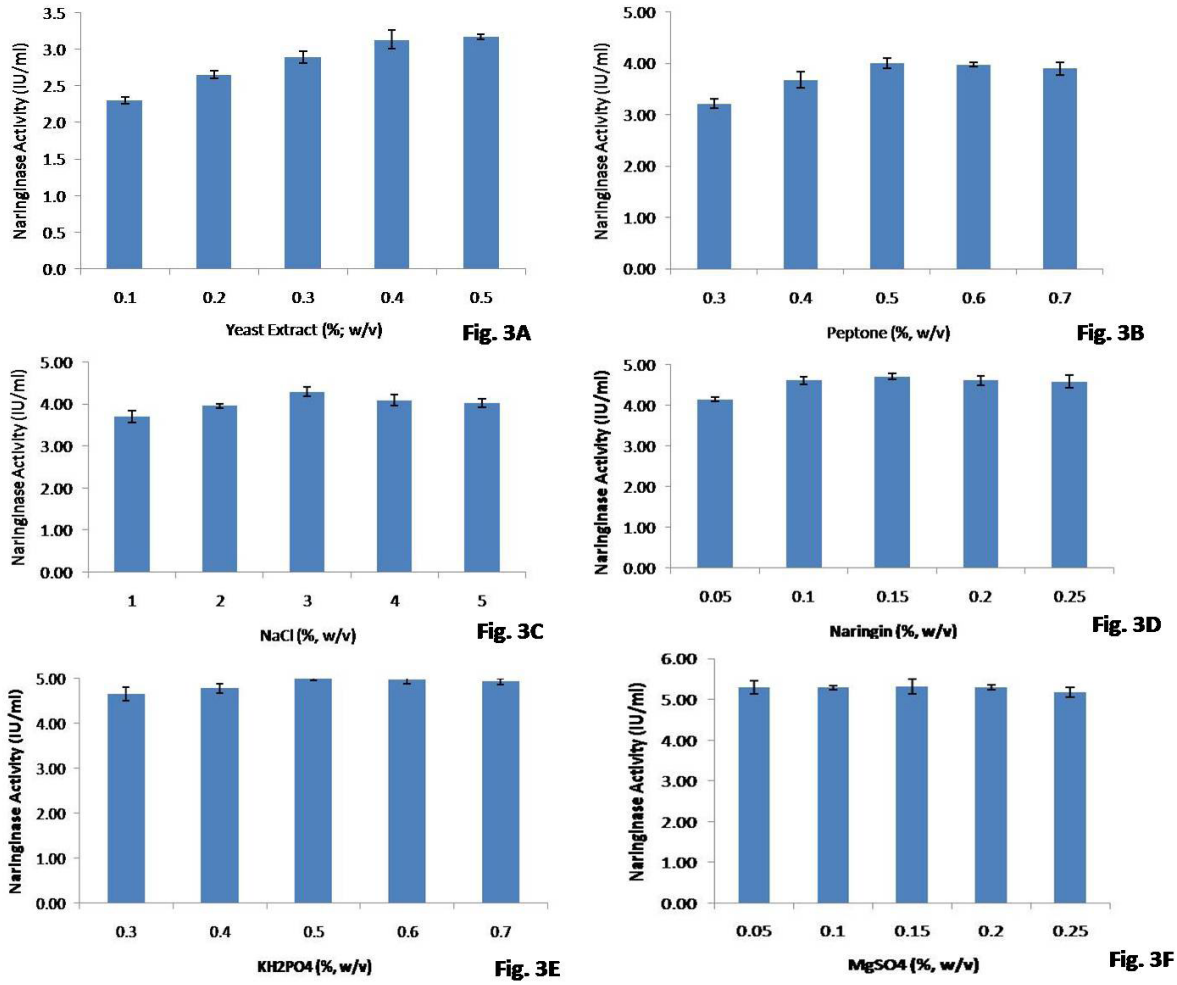

Figure 3: Effect of different concentrations of yeast extract (0.1%-0.5%, w/v)-Figure 3A, peptone (0.3%-0.7%, w/v)-Figure 3B, NaCl (0.3%-0.7%, w/v)-Figure 3C, naringin (0.05-0.25%; w/v)-Figure 3D, KH_2PO_4 (0.3%-0.7%, w/v)-Figure 3E and $MgSO_4$ (0.05%-0.25%, w/v)-Figure 3F on naringinase production. The media components were found to affect the production of naringinase in concentration dependent manner.

In an important study, Puri *et al*.[24] investigated the effect of various nitrogen and carbon sources, metal ions and process parameters on production of naringinase by *Staphylococcus xylosus* MAK2. Further, authors optimized

the level of Citrus Peel Powder (CPP) and observed enhanced enzyme production due to addition of CPP (Citrus Peel Powder) in the optimized medium.[24]

Citrus Peel Powder (CPP) as raw substrate for naringinase production

Peel powder of four citrus fruits (lemon, sweet lemon or mosambi, orange and kinnow; 1.0%, w/v) was investigated for production of naringinase at shake flask level. The citrus peel powder was used as substitute for naringin. All other media constituents were used at their optimal concentrations. The citrus peel powder supported the production of naringinase, though the production was low as compared to naringin (Figure 4). Further, sweet lemon or mosambi resulted in better production of naringinase (4.24 IU/mL) as compared to lemon, orange and kinnow peel powder (Figure 4). Earlier reports have described the production of naringinase using citrus peel powder as substrate. Borkar *et al*.[41] reported the fermentative production of naringinase from *Aspergillus niger* van Tieghem MTCC 2425 by using citrus waste peel. Srikantha *et al*.[33] have reported optimal production of naringinase from *Aspergillus flavus* using citrus peel as base support by solid state fermentation. Puri *et al*.[24] investigated the effect of citrus peel on production of naringinase from *Staphylococcus xylosus* MAK2.

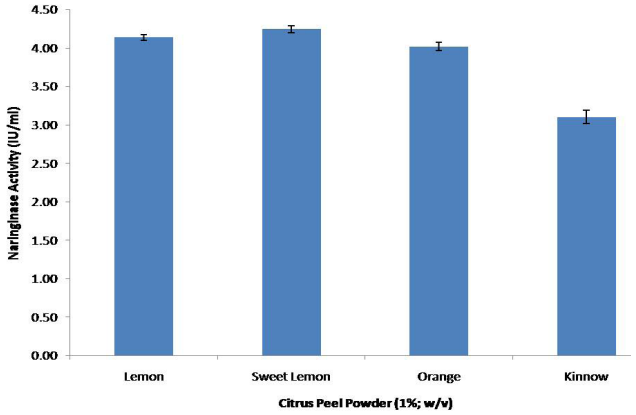

Figure 4: Effect of various citrus peel powders (1%; w/v) on naringinase production by *Paenibacillus stellifer* RAMCM-44. Peel powder of sweet lemon lead to higher production of naringinase as compared to lemon, orange and kinnow.

DISCUSSION

Microbes are important source of industrial enzymes and metabolites. Soil is well known for hosting a large number of microbes and considered as the main source for isolation of useful microbial strains. The diversity and number of microbes present in a specific area of soil depends upon different factors. Isolation of different naringinase producing microbial strains has been reported earlier by various researchers and reportedly some strains have been isolated from various sources including soil,[32] spoiled citrus peel[33] and other samples.[34] Soil rhizosphere hosts a large number of microbes which generally remain in association with roots of plants. The citrus fruits are natural source of naringin, a substrate for naringinase enzyme. The soil samples from rhizosphere of citrus plants were selected for isolation of naringinase producing microbial strains. Promising naringinase producing microbial strains were found to be present in the soil samples. Soil samples from citrus plants rhizosphere may be used further to isolate the efficient naringinase producing microbial strains.

Earlier, isolation of different bacterial strains belonging to genus *Paenibacillus* have been reported from various environmental samples and majority of them has been reported from soil. In soil, these strains have also been found in association with plant roots.[35] Few species of *Paenibacillus* have been reported earlier to produce useful antimicrobial compounds[35] and also enzymes having potential applications in various fields. The bacteria *Paenibacillus stellifer* has also been investigated for promotion of plant growth and for alleviating salt stress.[36] The *Paenibacillus stellifer* has also been isolated form paperboard and reported to produce cyclodextrin.[37] In current study, the *Paenibacillus stellifer* RAMCM-44 has been isolated from soil surrounding the citrus plant roots and have been found promising for production of naringinase. Further, the bacterial strain was used to produce naringinase at shake-flask level and optimization of media components lead to higher production of enzyme. The isolated *Paenibacillus stellifer* have also been found to produce naringinase in response to Citrus Peel Powder (CPP). Earlier reports have described the production of naringinase using citrus peel powder as substrate. Borkar *et al*.[41] reported the fermentative production of naringinase from *Aspergillus niger* van Tieghem MTCC 2425 by using citrus waste peel. Srikantha *et al*.[33] have reported optimal production of naringinase from *Aspergillus flavus* using citrus peel as base support by solid state fermentation. Puri *et al*.[24] investigated the effect of citrus peel on production of naringinase from *Staphylococcus xylosus* MAK2. The peel of citrus various citrus fruits are generally regarded as waste and have potential to be used as cost effective substrate. Our preliminary trial indicates that *Paenibacillus stellifer* RAMCM-44 have potential to hydrolyze the naringin in both purified form as well as from citrus peel, therefore, further studies may provide important insights into the production of naringinase from *Paenibacillus stellifer* RAMCM-44. This study may

broaden the exisiting knowledge about the *Paenibacillus stellifer* and its potential to be used for further research related aspects.

CONCLUSION

Microbes are considered as an important source of metabolites and enzymes of industrial importance. Naringinase is an important enzyme capable of hydrolyzing naringin into naringenin. Naringin is chiefly responsible for bitterness in citrus juices while naringenin is tasteless. Therefore, naringinase has significant applications in food industries particularly in de-bittering of citrus juices. Various bacterial and fungal strains have been studied for production naringinase. In current studies, naringinase producing bacterial strain has been isolated from citrus plant rhizosphere and characterized as *Paenibacillus stellifer* RAMCM-44 by 16S rRNA gene sequence followed by phylogenetic tree analysis. Further, media components have been optimized at shake-flask level using *one-variable-at-a-time* method. Naringinase production was also studied using citrus peel powder as raw source of naringin and promising production of naringinase have been obtained. This is first report on naringinase production from *Paenibacillus stellifer* and may be useful for further insights in to the group *Paenibacillus* sp.

CONFLICT OF INTEREST

The authors do not declare any conflict of interest.

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ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool; **NCBI:** National Center for Biotechnology Information; **CPP:** Citrus Peel Powder.

SUMMARY

In current study, naringinase producing bacterial stain was isolated from rhizosphere and characterized to be *Paenibacillus stellifer* RAMCM-44 by 16S rRNA gene sequence. The strain was efficient in naringinase production at shake-flask level. The strain was also found to be efficient for utilization of citrus peel powder as raw substrate. The media used for production of naringinase was optimized by *one-variable-at-a-time* method and this optimization lead to higher naringinase production (5.29 IU/mL) as compared to initial production (2.8 IU/mL).This is first report on naringinase production from *Paenibacillus stellifer* and open the way of exploring *Paenibacillus* sp. for further studies.

REFERENCES

1. Hasegawa S. Limonin bitterness in citrus juices. InFlavor chemistry: Thirty years of progress 1999 (pp. 89-106). Boston, MA: Springer US.
2. Yadav M, Sehrawat N, Sharma AK, Kumar V, Kumar A. Naringinase: microbial sources, production and applications in food processing industry. The Journal of Microbiology, Biotechnology and Food Sciences. 2018;8(1):717.
3. Awad GE, Abd El Aty AA, Shehata AN, Hassan ME, Elnashar MM. Covalent immobilization of microbial naringinase using novel thermally stable biopolymer for hydrolysis of naringin. 3 Biotech. 2016;6:1-0.
4. Yusof S, Ghazali HM, King GS. Naringin content in local citrus fruits. Food Chemistry. 1990;37(2):113-21.
5. Puri M, Banerjee A, Banerjee UC. Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344. Process Biochemistry. 2005;40(1):195-201.
6. Bodakowska-Boczniewicz J, Garncarek Z. Immobilization of naringinase from *Penicillium decumbens* on chitosan microspheres for debittering grapefruit juice. Molecules. 2019;24(23):4234.
7. Muñoz M, Holtheuer J, Wilson L, Urrutia P. Grapefruit debittering by simultaneous naringin hydrolysis and limonin adsorption using naringinase immobilized in agarose supports. Molecules. 2022;27(9):2867.
8. Chandler BV, Nicol KJ. Some relationships of naringin: their importance in orange juice bitterness. CSIRO Food Res. Quart. 1975;35:79-88.
9. Habelt K, Pittner F. A rapid method for the determination of naringin, prunin and naringenin applied to the assay of naringinase. Analytical biochemistry. 1983;134(2):393-7.
10. Ribeiro MH. Naringinases: occurrence, characteristics and applications. Applied microbiology and biotechnology. 2011;90:1883-95.
11. Puri M, Marwaha SS, Kothari RM, Kennedy JF. Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering. Critical reviews in biotechnology. 1996;16(2):145-55.
12. Falch EA. Industrial enzymes-developments in production and application. Biotechnology advances. 1991;9(4):643-58.
13. Kumar A, Yadav M, Tiruneh W. Debarking, pitch removal and retting: role of microbes and their enzymes. Physical Sciences Reviews. 2020;5(10):20190048.
14. Kumar S, Panwar P, Sehrawat N, Upadhyay SK, Sharma AK, Singh M, Yadav M. Oxalic acid: recent developments for cost-effective microbial production. Physical Sciences Reviews. 2024; 9:891-907.
15. Chauhan S, Mitra S, Yadav M, Kumar A. Microbial production of lactic acid using organic wastes as low-cost substrates. Physical Sciences Reviews. 2024; 9:875-89.
16. Yadav M, Sehrawat N, Kumar S, Sharma A, Singh M, Kumar A. Malic acid: fermentative production and applications. Physical Sciences Reviews. 2024;9(1):187-99. https://doi.org/10.1515/psr-2022-0165
17. Mukesh Y, Nirmala S, Amit K. Microbial laccases in food processing industry: current status and future perspectives. Research Journal of Biotechnology. 2018;13(9):108-13.
18. Singh R, Singh R, Yadav M. Molecular and biochemical characterization of a new endoinulinase producing bacterial strain of *Bacillus safensis* AS-08. Biologia. 2013;68(6):1028-33.
19. Singh RS, Yadav M. Enhanced production of recombinant aspartase of *Aeromonas media* NFB-5 in a stirred tank reactor. Bioresource technology. 2013;145:217-23.
20. Singh R.S. & Yadav M. (2012). Biochemical and molecular characterization of a new aspartase producer *Aeromonas media* NFB-5 from effluent of a fertilizer factory. *Current Biotechnology* 1:185-93.

21. Malik K, Sehrawat N, Tripathi A, Nara R, Beniwal M, Singh M, Kumar S, Yadav M. Molecular characterization of yellow pigment producing bacterial strain as *Kocuria flava* KMSM-18 and fermentative production of yellow pigment. Indian Journal of Natural Sciences. 2023. 14(81):66790-7.
22. Puri M, Kaur A Molecular identification of *Staphylococcus xylosus* MAK2. Appl Biochem Biotechnol 2010;162:181-91
23. Radhakrishnan I, Sampath S, Kumar S. Isolation and characterization of enzyme naringinase from *Aspergillus flavus*. International journal of advanced biotechnology and research. 2013;4(2):208-12.
24. Puri M, Kaur A, Barrow CJ, Singh RS. Citrus peel influences the production of an extracellular naringinase by *Staphylococcus xylosus* MAK2 in a stirred tank reactor. Applied microbiology and biotechnology. 2011;89:715-22.
25. Patil SV, Koli SH, Mohite BV, Patil RP, Patil RR, Borase HP, Patil VS. A novel screening method for potential naringinase-producing microorganisms. Biotechnology and applied biochemistry. 2019;66(3):323-7.
26. Davis WB. Determination of flavonones in citrus fruits. Anal Chem 1947;19:476.
27. Puri M, Kaur A, Singh RS, Singh A. Response surface optimization of medium components for naringinase production from *Staphylococcus xylosus* MAK2. Applied biochemistry and biotechnology. 2010;162:181-91.
28. Pavithra, M, Prasanna DB, Saidutta MB. Production of naringinase by a new soil isolate of Serratia sp.: effect of different carbon and nitrogen sources. Research Journal of Biotechnology, 2012;7:208-11.
29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10. https://doi.org/10.1016/S0022-2836(05)80360-2.
30. Thompson JD, Gibson TJ, Higgins DG. Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics. 2003;2-3. https://doi.org/10.1002/0471250953.bi0203s00.
31. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4(4):406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
32. Kumar A, Singh MK, Amena SY. Optimization of naringinase production and its purification from *Micrococcus* sp. Int J Pharm Pharm Sci. 2015;7(2):269-72.
33. Srikantha K, Kapilan R, Seevaratnam V. Characterization of best naringinase producing fungus isolated from the citrus fruits. Int. J. Biol. Res. 2016;4:83
34. Thammawat K, Pongtanya P, Juntharasri V, Wongvithoonyaporn P. Isolation, preliminary enzyme characterization and optimization of culture parameters for production of naringinase isolated from *Aspergillus niger* BCC 25166. Agriculture and Natural Resources. 2008;42(1):61-72.
35. Grady EN, MacDonald J, Liu L, Richman A, Yuan ZC. Current knowledge and perspectives of *Paenibacillus*: a review. Microbial cell factories. 2016;15:1-8.
36. Gopal NO, Anandham R. Plant growth promotion and alleviation of salts in rice by *Paenibacillus* spp. Journal of Pharmacognosy and Phytochemistry. 2020;9(4):1485-9.
37. Suominen I, Sproer C, Kampfer P, Rainey FA, Lounatmaa K, Salkinoja-Salonen M. *Paenibacillus stellifer* sp. nov., a cyclodextrin-producing species isolated from paperboard. International journal of systematic and evolutionary microbiology. 2003;53(5):1369-74.
38. Mahto RB, Yadav M, Sasmal S, Bhunia B. Optimization of process parameters for production of pectinase using *Bacillus subtilis* MF447840. 1. Recent patents on biotechnology. 2019;13(1):69-73.
39. Mahto RB, Yadav M, Muthuraj M, Sharma AK, Bhunia B. Biochemical properties and application of a novel pectinase from a mutant strain of *Bacillus subtilis*. Biomass Conversion and Biorefinery. 2023;13(12):10463-74.
40. Chen D, Niu T, Cai H. Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316. African Journal of Biotechnology. 2010;9(31):4970-8.
41. Borkar V, Chakraborty S, Gokhale JS. Fermentative production of naringinase from *Aspergillus niger* van Tieghem MTCC 2425 using citrus wastes: Process optimization, partial purification and characterization. Applied Biochemistry and Biotechnology. 2021;193(5):1321-37.

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