Isolation, Production Optimization, and Purification of a Debittering Enzyme from *Bacillus megaterium* AULS 1

G. Nandhini Devi,[*] **G. Sangavi,**
M. Chakravarthy, and J. Louis Sweetlin
Centre for Food Technology,
Department of Biotechnology,
Anna University, Chennai

Bitterness in citrus juice is a common quality issue in the fruit-based beverage industry. Naringin, the dominant flavonoid responsible for bitterness in citrus fruit juice, can be mitigated by the enzyme naringinase. This study focuses on the isolation of novel naringinase-producing bacterium from mandarin orange peel, followed by the production and partial purification of naringinase. The bacterial strain exhibiting the highest enzyme production potential was identified as *Bacillus megaterium* AULS 1. The effects of carbon source (naringin) concentration, inducer (naringenin), pH, and temperature on naringinase production were investigated. Optimized conditions yielded a maximum naringinase production of 386.43 U mL^{-1}. The produced enzyme was partially purified using ammonium sulfate precipitation and dialysis. The effects of pH, temperature, and metal ion concentration on the activity of the partially purified enzyme were evaluated, resulting in a highest activity of naringinase of 539.25 U mL^{-1} under optimized conditions. The debittering activity of the partially purified enzyme was 45.78 %. These findings demonstrate that *Bacillus megaterium* AULS 1 has the potential for naringinase production, and can be utilized for debittering citrus juices.

Keywords
naringinase, debittering, *Bacillus megaterium*, naringin, citrus juice

Introduction

Natural fruit juices are an important source of essential nutrients and contain various bioactive components such as antioxidants, polyphenols, and fiber, which are vital for human health[1]. Citrus fruits, such as sweet orange, mandarin, lemon, acid lime, and, grapefruit are produced in huge quantities in India, which ranks 3rd in worldwide citrus fruit production[2]. These fruits are nutritious and rich in bioactive compounds such as carotene, ascorbic acid, folic acid, and flavonoids, which possess antioxidant and immunomodulatory properties[3]. Flavonoids play a significant role in the quality of fruit juices by influencing taste and appearance. Naringin (4,5,7-trihydroxyflavonone-7-rhamnoglucoside), a flavonoid present in citrus fruits, imparts characteristic bitterness to the juices[4]. Bitterness is a major limiting factor in commercial acceptance of citrus fruit juices[5]. Reduction of naringin can be achieved through various technologies such as adsorptive debittering, chemical methods, treatment with resins and β-cyclodextrin. However, these technologies have been reported to have numerous drawbacks and limitations, including altering the composition and organoleptic properties of the juices[6]. An efficient method for debittering citrus juices is through enzymatic treatment, which is a promising technique for industrial applications[7].

Naringinase (EC 3.2.1.40) is an enzyme complex containing α-L-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21) activities[8]. The hydrolysis of naringin is a two-step reaction. Initially, the naringinase enzyme hydrolyzes naringin into L-rhamnose and prunin via α-L-rhamnosidase activity, followed by the hydrolysis of prunin into naringenin and D-glucose via β-glucosidase activity[9]. Naringenin (4,5,7-trihydroxyflavonone), the end product of this hydrolysis reaction is non-bitter in nature and cannot be reconverted to naringin[5]. The schematic representation of naringin hydrolysis by naringinase enzyme is provided in Fig. 1.

Naringinase enzyme is widely distributed in nature and has been found in plants, yeasts, fungi, and bacteria[10]. Several microorganisms have been screened for the production of naringinase to enhance yield and reduce the enzyme production costs[11]. Microorganisms reported in the literature for production of naringinase include *Aspergillus niger, Aspergillus sojae, Penicillium* sp., *Penicillium*

---

[*]Corresponding author: Dr. G. Nandhini Devi, Professor,
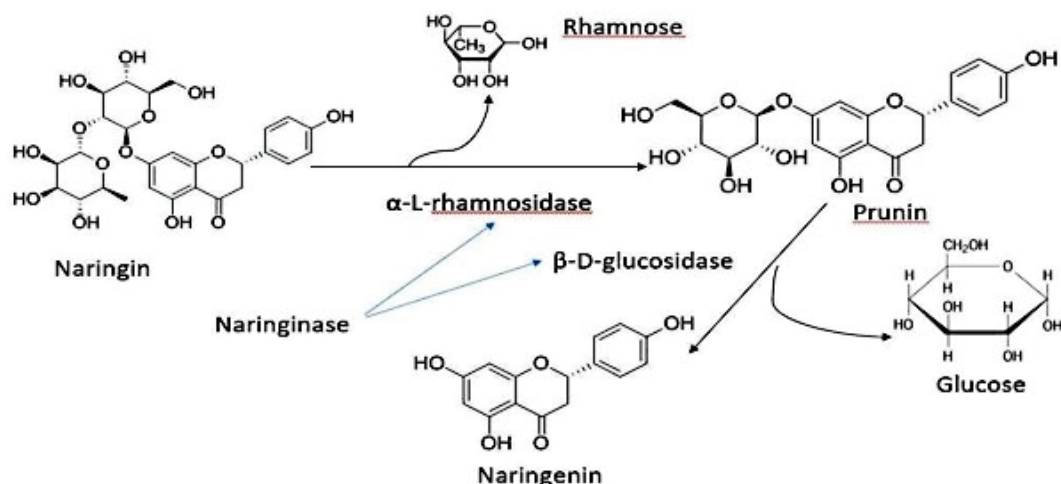E-mail address: projectsagnlabs@gmail.com

F i g . 1 – *Hydrolysis of naringin by naringinase*

decumbens, Rhizopus nigricans[10]. The production of naringinase from fungi is well-documented, with *Aspergillus niger* being particularly noted for industrial applications[12]. However, reports on naringinase production by bacteria are limited in the literature[13]. Bacteria known to produce naringinase include *Bacillus methylotrophicus*[9], *Serratia* sp.[14], *Staphylococcus xylosus* MAK2[15], *Bacillus cereus*[16], *Micrococcus* sp.[17], and *Psidium guajava* L.[18] In this study, a novel naringinase-producing bacterium was isolated from citrus fruit peel. The process parameters for enhancing naringinase production from the isolated strain were optimized, followed by partial purification and characterization of the enzyme. The debittering efficiency of the produced enzyme was also evaluated.

Materials and methods

Materials

Naringin (assay ≥95 %) and naringenin standard (assay ≥ 97 %) were procured from Sigma. The media used for isolation of bacteria were procured from Hi-media (Mumbai, India). The Bradford reagent was purchased from Bio-Rad. Solvents used for HPLC were procured from Merck India. All the other chemicals and reagents used were of analytical grade.

Methodology

Collection of citrus fruit

Citrus fruit (mandarin orange – *Citrus reticulata*) was procured from a local market in Chennai, Tamil Nadu. The citrus fruit was washed with distilled water and the skin removed. The fruit peel was immersed in a saline solution for 24 h.

Screening and isolation of naringinase-producing bacteria

After 24 h of incubation, 1 mL of the saline solution was inoculated in 50 mL of selective enrichment broth containing naringin as the sole carbon source. The composition of the media was as follows (in g L^{-1}): 5.0 NH_4NO_3, 0.2 KCl, 0.4 KH_2PO_4, 0.01 $FeSO_4 \cdot 7H_2O$, 0.01 $ZnSO_4$, 0.01 $MnSO_4$, 0.2 $MgSO_4 \cdot 7H_2O$, and 1.0 naringin. The sample was then kept in an incubator shaker at 240 rpm and 30 °C for 24 h. After incubation, the sample was serially diluted and spread on nutrient agar plates, which were incubated at 37 °C for 24 h. Bacterial samples were picked and isolated as single colonies by streaking. The isolated individual pure cultures were stored on slant culture at 4 °C.

Primary screening (Ferric chloride test)

The naringinase-producing bacteria were qualitatively screened using the ferric chloride ($FeCl_3$) test. The pure bacterial culture was grown on selective enrichment medium agar plates. Color changes in the media were observed after reacting with ferric chloride. Plates with the bacterial strains that turned reddish-brown were selected for further analysis[19]. The selected cultures were inoculated into selective enrichment broth, and after 24 h of incubation, the media were centrifuged and the supernatant was collected for further analysis.

Secondary screening (Davis method)

Secondary screening quantified the naringinase enzyme activity using the Davis method[20]. The supernatant (0.1 mL) was equilibrated with 0.35 mL of acetate buffer (1 M, pH = 4), and then 0.65 mL of 0.01 % naringin was added to the mixture. The solution mixture was incubated at 50 °C for 60 min. After incubation, an aliquot (0.2 mL) was taken

from the solution mixture and mixed with 0.4 mL of diethylene glycol and 0.2 mL of 4 N NaOH. The yellow color developed was measured at 420 nm. The concentration of naringin was determined using a standard curve[21]. One unit (IU) of enzyme activity was defined as the amount of enzyme that could hydrolyze 1 μmol of naringin mL^{-1} per minute under the test conditions.

Identification of the naringinase strain

The taxonomy of the isolated strains was examined using Bergey's Manual of Determinative Biology[22] and confirmed by16S rDNA sequencing. Following the preliminary investigation, the growth characteristics of the isolates were analyzed. The plates were incubated at 30 °C for 30 h, and at the end of the incubation period, all the plates were examined for growth, and their colony characteristics were studied and noted. Slide cultures were used for microscopic observations to study the pattern of the isolate. Biochemical tests such as catalase, oxidase, and indole tests were conducted to understand the morphological characteristics of the strain. The catalase test was conducted using the slide method. In this method, a small amount of colony growth was placed on a dry glass slide using a loop, and a drop of 3 % hydrogen peroxide was added on the glass slide. Formation of oxygen bubble indicates a positive strain. The oxidase test was carried out by placing a small amount of colony growth on a filter paper using a loop, and adding 1 or 2 drops of 1 % Kovac's oxidase reagent on the organism smear. A color change to dark purple within 5 to 10 s indicates a positive strain. The indole test was conducted by placing several drops of indole spot reagent on a filter paper. Using an inoculating loop or wooden applicator stick, a portion of an isolated colony from a non-selective media was picked and rubbed onto the reagent-saturated area of the filter paper. Formation of pink to red color in the reagent layer on top of the medium within seconds of adding the reagent indicates a positive strain.

Microbial growth studies

The growth curve of the isolate *Bacillus megaterium* AULS 1 was analyzed in selective enrichment broth with naringin as the carbon source. The medium was incubated at 37 °C, and aliquots were collected every two hours. The optical density was measured at 600 nm.

Optimization of naringinase production

The production of naringinase from the selected isolate was optimized using selective enrichment media with naringin as a carbon source in shake flasks. The effect of the carbon source on naringi-

nase production was studied by varying the concentration of naringin from 50 mg L^{-1} to 200 mg L^{-1}. Similarly, effect of the inducer on naringinase production was studied by varying the concentration of naringenin from 0.02 mg L^{-1} to 0.1 mg L^{-1}. For both the experiments, the media pH was maintained at 6, and the samples were incubated at 37 °C. The effect of pH was studied by varying the pH from 5 to 8, and the effect of incubation temperature was analyzed by varying the temperature from 20 °C to 45 °C. For the optimization experiments, aliquots were collected at the optimized incubation time, centrifuged (12,500 g for 15 min at 4 °C), and the supernatant was collected for enzyme activity analysis.

Partial purification of naringinase

The culture was grown under optimized conditions and, after the incubation period, it was centrifuged (12,500 g, 15 min, 4 °C) and the supernatant was collected. Saturated ammonium sulfate was added to the supernatant at varying concentrations (30 %, 50 %, and 80 % saturation) with continuous stirring to precipitate the protein. The sample was then incubated at 4 °C for 24 h. After incubation, the sample was centrifuged, and the resulting pellet was dissolved in 10 mM Tris-HCl buffer (pH 8) and dialyzed with the same buffer for 12 h at 4 °C [23]. The protein content was estimated using the Bradford assay.

Molecular weight determination by polyacrylamide gel electrophoresis (PAGE)

The partially purified naringinase was subjected to SDS PAGE analysis using 4 % and 10 % (v/v) acrylamide gels of stacking and separating gel. The gel was loaded and run for 4 h at 60 V, then the gel was cut and stained with Coomassie Brilliant Blue (CBB-R 250) for 4 h, followed by overnight destaining visualize the protein band. The molecular weight was calculated by comparing the protein band to a standard protein marker[24].

Characterization of partially purified naringinase

The effect of pH on naringinase activity was studied by adjusting pH of the naringinase enzyme using sodium acetate and phosphate buffer. The pH-adjusted enzyme was incubated, and samples were withdrawn after incubation time and analyzed for enzyme activity using the Davis method. Similarly, the enzyme was incubated at temperatures from 27 °C to 47 °C at 5 °C intervals at the predetermined pH, to determine the effect of temperature on enzyme activity. The effect of metal ions on enzyme activity and stability was determined by adding metal ions (Fe^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}) at different concentrations (5 mM, 10 mM, and 15 mM)

to the partially purified naringinase enzyme, and the activity of the enzyme was determined after incubation.

Determination of debittering efficiency of naringinase in citrus juice

To analyze the debittering efficiency, fresh citrus juices (lemon and mandarin orange) were prepared by blending the fruits in a blender and pressing the resulting pulp through four layers of gauze to collect the juice. The fresh juices (4 mL) were diluted with water (1 mL), and partially purified naringinase enzyme was added. The mixture was incubated at preoptimized conditions. After incubation, the reaction mixture was centrifuged, and the supernatant was collected and filtered using a 0.22 μm PTFE filter. The filtrate was used for HPLC analysis.

The debittering efficiency of the enzyme was determined by quantifying the concentration of naringin and naringenin using HPLC (Perkin Elmer 250) coupled with a C-18 Zorbax Eclipse XDB column (4.6×150 mm, 5 μm particle size, 50 °A pore size). Isocratic elution was performed using the mobile phase (11.4 % methanol, 26.6 % acetonitrile, and 62 % deionized water) at a flow rate of 0.4 mL min^{-1}. The column temperature was maintained at 30 °C, and the compounds were detected at 280 nm. The concentration of naringin and naringenin was determined using a standard curve.

Results and discussion

Isolation and identification of naringinase-producing bacteria

Naringinase-producing bacteria were isolated from citrus fruit peel using selective enrichment media with naringin as the sole carbon source. Four colonies with different morphological features were screened using nutrient media and labelled as AULS

1 to AULS 4. The four bacterial strains were primarily screened for naringinase production using selective enrichment agar plates with 1 % FeCl$_3$. Bacteria-producing naringinase enzyme hydrolyze the naringin in the agar plates into naringenin, which reacts with ferric chloride to produce a reddish-brown color[19]. All four bacterial isolates showed positive response to the ferric chloride test. After qualitative screening, the naringinase activity of the isolates were estimated using the Davis method. The bacterial isolate AULS 1 exhibited the highest naringinase production compared to the other isolates and was selected for further studies. The bacterial isolate AULS 1 was found to be gram-positive and rod-shaped. Biochemical characterization showed the strain is catalase-positive, oxidase-negative and indole-negative. The strain AULS 1 was identified as *Bacillus megaterium* by 16S rDNA sequencing. Phylogenetic analysis revealed that isolate AULS 1 is closely related to *Bacillus* sp. (Fig. 2). The 740 bp 16S rDNA sequence of the isolate has been submitted to the Genbank database with the accession number MW145001.

Microbial growth

The growth curve of the isolate *Bacillus megaterium* AULS 1 is presented in Fig. 3. The log phase in the selective enrichment medium peaked at 30 h. Naringin in the medium was metabolized by the bacteria for naringinase production. After 30 h, the amount of naringin was depleted, which was reflected in the decline in bacterial growth.

Optimization of growth media for naringinase production

Growth media conditions, such as the concentration of carbon source (naringin) and inducer (naringenin), pH, and incubation temperature, were optimized to enhance the production of naringinase enzyme.
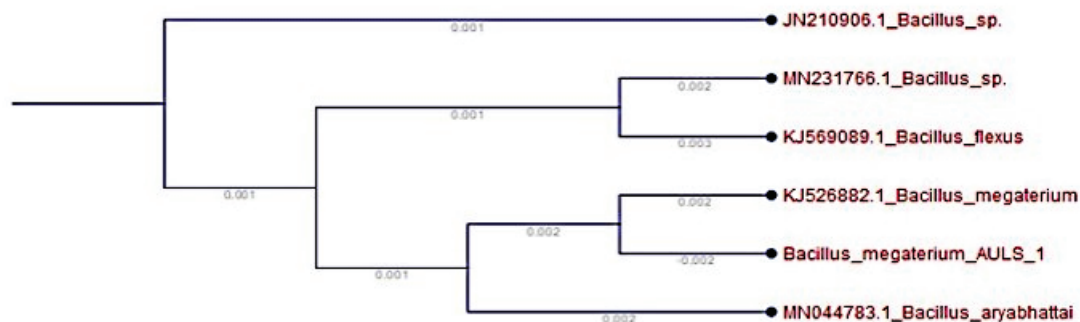

F i g . 2 – *Phylogenetic tree based on the 16S rDNA sequences of Bacillus megaterium AULS 1 and related bacteria, constructed using the neighbour-joining method*
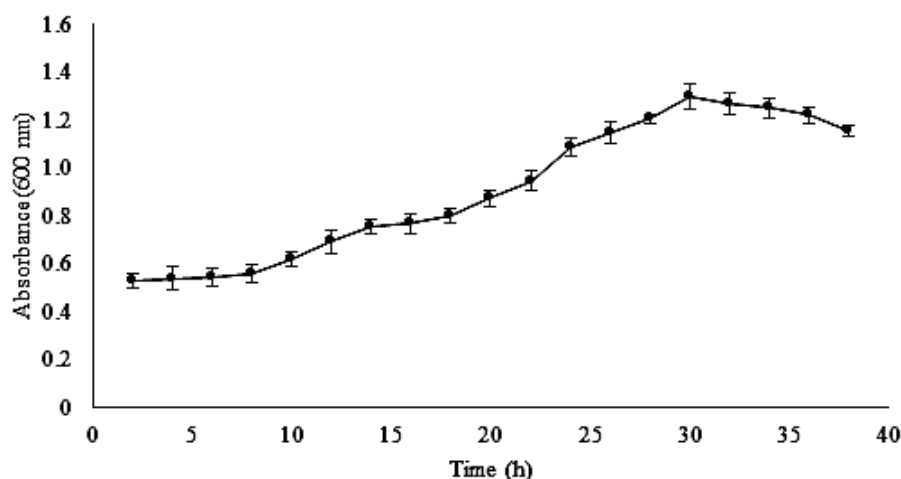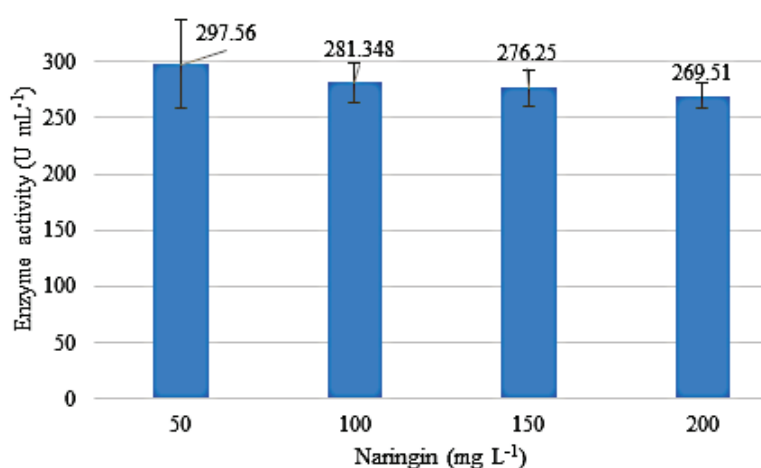
Fig. 3 – *Growth curve of Bacillus megaterium AULS 1*

Fig. 4 – *Effect of naringin concentration on enzyme production*

Effect of carbon source and inducer on naringinase production and activity

The effect of varying the carbon source on naringinase production, in terms of enzyme activity, is presented in Fig. 4. The highest enzyme production of 297.56 U mL^{-1} was observed at a naringin concentration 50 mg L^{-1}. Further increases in naringin concentration resulted in decreased enzyme production. Various carbon sources have been studied in the literature, and the effect of carbon source on naringinase production varied according to the microorganisms and conditions studied.

For example, a study on the effect of the carbon sources (naringin, rhamnose, and molasses) for optimization of production by *Aspergillus niger* ATCC1015 reported rhamnose as the best carbon source[25]. Another study optimizing naringinase production by *Serratia* sp. used glucose, rhamnose, and sucrose as carbon sources, with glucose yielding the highest naringinase production[14]. Naringinase production by *Aspergillus oryzae* JMU316 was optimized using the following carbon sources – pomelo pericarp powder, lactose, rhamnose, sucrose, maltose, glucose, and pomelo juice. Naringin as carbon source was used as a control for the study. Results showed that the naringinase specific activity was higher when naringin was used as the carbon source[26]. Naringin used as an inducer demonstrated the highest naringinase production (7.46 U L^{-1}) in *Bacillus methylotrophicus*[9]. The concentration of the inducer is significant for enzyme production. The effect of varying the inducer concentration on naringinase production is shown in Fig. 5. Maximum enzyme production of 304.84 U mL^{-1} was observed at a naringenin concentration of 0.04 mg L^{-1}. Similar results were reported for naringinase production by *S. xylosus* MAK2 when citrus peel powder was used as an inducer, with enzyme production increasing when the concentration of citrus peel powder increased up to 2 % (w/v), before decreasing with further increases[13]. In naringinase production by *Bacillus methylotrophicus*, naringenin exhibited the second-highest production of naringinase
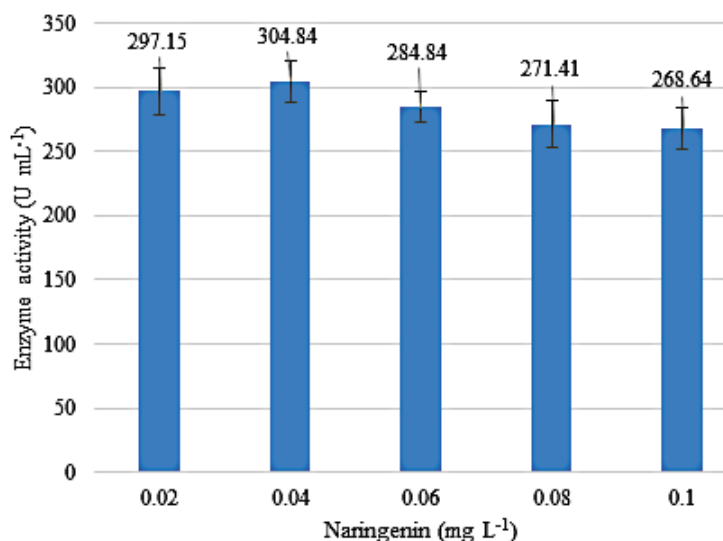
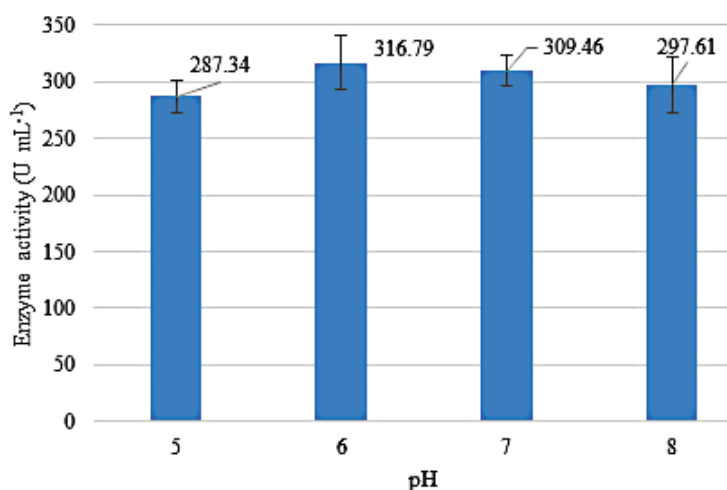F i g . 5 – *Effect of naringenin concentration on enzyme production*
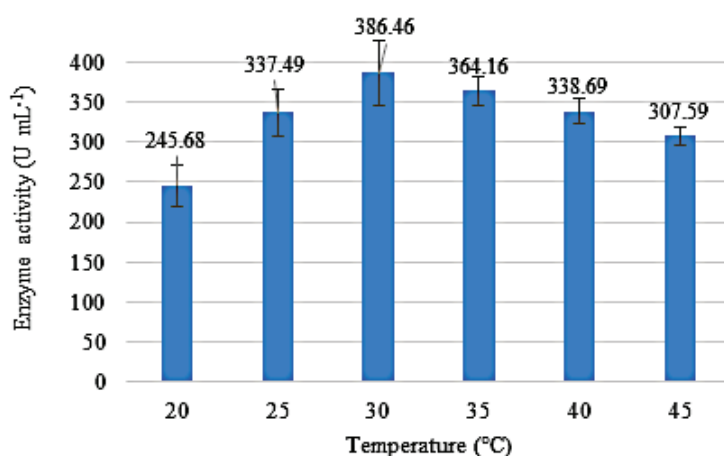

F i g . 6 – *Effect of pH on enzyme production*

F i g . 7 – *Effect of temperature on enzyme production*

(5.74 U L^{-1}) [9]. Naringenin was used as an inducer for production of naringinase by *Aspergillus niger* MTCC 1344 [27]. The effect of inducers (naringin, naringenin, rutin, and hesperidin) on enzyme produc-

tion by *Aspergillus niger* VB07 showed both naringin and naringenin effective in enzyme production[28].

Effect of pH on naringinase production and activity

The optimal pH for naringinase production was found to be pH 6.0, with a naringinase activity of 316.79 U mL^{-1} (Fig. 6). Similar results have been reported for naringinase production in *Bacillus methylotrophicus*[9] and *Aspergillus niger* VB07 [28]. The optimal pH range for naringinase production is reported to be pH 4–6 [29]. Acidic pH conditions (pH<4) are not suitable for supporting biomass production and enzyme synthesis[9]. The pH of the medium is a critical parameter in bacterial growth, enzyme production, and the release of extracellular enzymes. An increase in the medium pH above 6 resulted in biomass reduction and decreased enzyme activity (Fig. 6).

Effect of temperature on naringinase production and activity

The highest naringinase production for *Bacillus megaterium* AULS 1 was observed at 30 °C, with an enzyme activity of 386.46 U mL^{-1} (Fig. 7). Above 30 °C, a decline in enzyme activity was noted, likely due to changes in cell metabolism at elevated temperatures. The highest enzyme production for *Bacillus methylotrophicus* of 8 U L^{-1} was also obtained at 30 °C [9]. Similarly, an optimal temperature of 30 °C was reported for naringinase production from *Staphylococcus xylosus* MAK2 [13].

Partial purification of naringinase

The naringinase produced by *Bacillus megaterium* AULS 1 was partially purified by ammonium sulfate precipitation (30 %, 50 %, and 80 % saturation) followed by dialysis. Ammonium sulfate precipitation yielded naringinase with a specific activity of 1837.38 U mg^{-1} and a purification fold of 4.02. Further purification by dialysis yielded naringinase with a specific activity of 2246.12 U mg^{-1}, and a purification fold of 4.91 (Table 1). The specific activity of naringinase from *Aspergillus niger* after purification by 50 % ammonium sulfate and gel filtration was reported as 355.40 U mg^{-1} and 7279.37 U mg^{-1} respectively[30]. Similarly, purification of naringinase from *Bacillus amyloliquefaciens* 11568 by ammonium sulfate precipitation (40–80 %) yielded an enzyme with a specific activity of 982 U mg^{-1} [23].

Characterization of partially purified naringinase

The partially purified enzyme appeared as a single band on an SDS-PAGE gel, with an estimat-

T a b l e 1 – *Purification of extracellular naringinase from Bacillus megaterium AULS 1*

Enzyme sample	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold
Crude extract	1000	733.0	334860	456.834	1.00
30 % (NH$_4$)$_2$SO$_4$ precipitation	850	391.96	338075	862.512	1.89
50 % (NH$_4$)$_2$SO$_4$ precipitation	540	204.95	259114	1264.27	2.77
80 % (NH$_4$)$_2$SO$_4$ precipitation	320	84.76	155737	1837.38	4.02
Dialysis	80	19.02	42734	2246.12	4.91

ed molecular weight (MW) of 85 kDa. The MW of naringinase *Bacillus amyloliquefaciens* 11568 was reported to be 32 kDa, also appearing as a single band on SDS – PAGE[23]. The MW of *Micrococcus* sp. was determined to be 48 kDa [17]. In the literature, the molecular masses of naringinases have been reported to range from 70 to 240 kDa [5]. This suggests that the naringinase enzyme exhibits a wide range of molecular weights that vary depending on its source of synthesis. The molecular weight was confirmed using mass marker proteins with the partially purified naringinase.

Effect of pH, temperature and metal ions on enzyme activity

The optimal pH of the naringinase enzyme was found to pH 5.5 (Fig. 8). The optimal pH for naringinase activity is highly dependent on the microbial
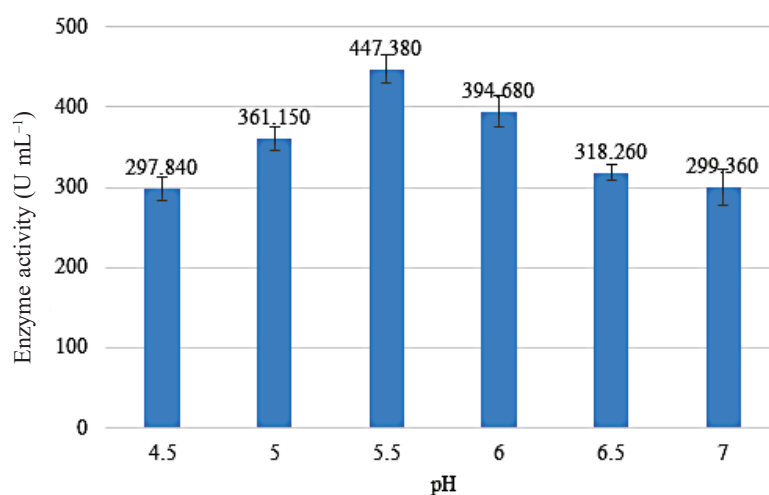

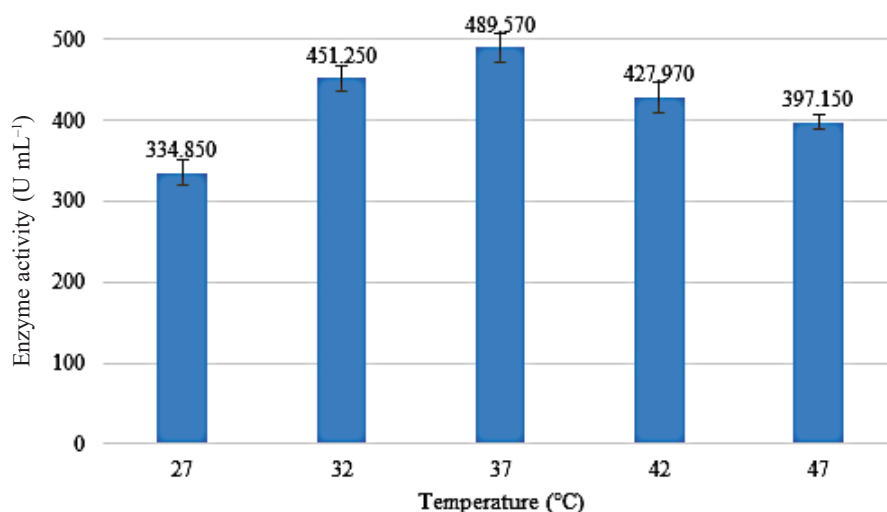F i g . 8 – *Effect of pH on enzyme activity*

F i g . 9 – *Effect of temperature on enzyme activity*

source, with reported optimal pH ranges from 4.2 – 6.5 [6]. In this study, the enzyme remained stable across the studied pH conditions and retained activity in acidic conditions, suggesting its suitability for hydrolyzing naringin in acidic citrus juices. The optimal pH reported in this study is similar to literature reports[5,9,31,32]. The optimal temperature for naringinase activity was found to be 37 °C. While enzyme activity increased with temperature, it declined after 40 °C (Fig. 9). Like pH, the optimal temperature for naringinase activity varied with microbial sources. For example, the optimal temperature for narignase from *Bacillus methylotrophicus* and *Aspergillus niger* was reported to be 50 °C [9,30]. The naringinase from *Aspergillus sojae* was found to be highly stable at 37 °C [7]. The effect of metal ions on the naringinase activity is presented in Table 2. In this study, increasing concentrations of Mg^{2+} and Mn^{2+} improved the naringinase activity, while Fe^{2+} reduced it. Increasing concentrations of Cu^{2+} and Ca^{2+} up to 10 mM improved enzyme activity, but activity decreased at 15 mM. Naringinase produced from *Aspergillus oryzae* 11250 was reported to be inhibited by Mn^{2+}, Pb^{2+}, Cu^{2+} and EDTA at concentrations up to 10 mM, with Fe^{2+} and Zn^{2+} showing slight inhibition[33]. These varying effects are due to the metal ions binding at different sites of the enzyme, causing conformational changes in the enzyme.

Debittering efficiency of naringinase

Naringinase enzyme effectively hydrolyzed naringin in both lemon and mandarin orange juice (Table 3). The initial concentration of naringin in lemon juice was 146.26 μg mL⁻¹, which reduced to 79.32 μg mL⁻¹ after enzyme activity. In mandarin orange juice, the naringin concentration reduced from 56.35 μg mL⁻¹ to 32.29 μg mL⁻¹. The debittering efficiency of naringinase in lemon and mandarin orange juice was 45.78 % and 42.71 %, respectively.

These results suggest that naringinase from *Bacillus megaterium* AULS 1 can effectively remove the bitter taste of citrus juices. This effect is consistent with literature reports. For instance, a debittering efficiency of 56 % was achieved in kinnow mandarin juice using alginate-entrapped naringinase[34]. Naringinase produced from a novel *Bacillus amyloliquefaciens* 11568 isolate reduced the naringin concentration to less than 30 μg mL⁻¹ [23] and the debittering efficiency of *Penicillium purpurogenum* was reported to be 74 % [2]. However, the naringinase purified from *Penicillium* was found to be inefficient in debittering citrus juice[35]. Variations in debittering efficiency may be due to the use of different juices, enzyme concentrations, and optimized conditions.

Table 2 – *Effect of metal ions on naringinase activity*

Metal ions	Enzyme activity (U mL⁻¹)		
	5 mM	10 mM	15 mM
Cu^{2+}	215±12.956	224±11.025	219.62±16.266
Fe^{2+}	289.04±18.264	261.27±13.264	214.64±13.325
Mn^{2+}	315.21±19.794	325.84±4.26	349.648±9.36
Ca^{2+}	447.51±18.637	469.87±12.247	431.94±584
Mg^{2+}	494.25±16.235	539.25±18.364	521.79±19.21

Table 3 – *Debittering of citrus juice by isolated enzyme*

Sample	Naringin (fresh juice) (μg mL⁻¹)	Naringin (after hydrolysis (μg mL⁻¹)	Naringenin (fresh juice) (μg mL⁻¹)	Naringenin (after hydrolysis) (μg mL⁻¹)
Lemon	146.264	79.315	9.254	21.986
Mandarin orange	56.352	32.282	1.908	6.084

Conclusion

In this study, a novel extracellular debittering enzyme-producing bacterium, *Bacillus megaterium* AULS 1, was isolated from citrus fruit peel. Optimization of culture media conditions significantly improved naringinase production. The optimal conditions for maximum naringinase production (386.43 U mL⁻¹) were determined to be a naringin concentration of 50 mg L⁻¹, a naringenin concentration of 0.04 mg L⁻¹, a pH of 6, and incubation temperature of 30 °C. The produced enzyme was partially purified, and its activity was studied. The debittering analysis results indicated that the naringinase produced by *Bacillus megaterium* was effective in debittering fruit juices, suggesting its potential application in the debittering of fruit juices. Further optimization studies are anticipated to improve enzyme production and debittering efficiency.

References

1. *Upadhya, S. H., Ganavi, H., Veda, S., Thakur, M.,* Natural fruit sugars and human health: A reality, Adv. Nutr. Food Sci. **4** (2019) 1.

2. *Patil, M. B., Dhake A. B.,* Debittering of citrus fruit juice by naringinase of *Penicillium purpurogenum*, Int. J. Engg. Res. & Sci. & Tech. **3** (2014) 1. http://www.ijerst.com/currentissue.php

3. *Ribeiro, I. A., Rocha, J., Sepodes, B., Mota-Filipe, H., Ribeiro, M. H.,* Effect of naringin enzymatic hydrolysis towards naringenin on the anti-inflammatory activity of both compounds, J. Mol. Catal. B Enzym. **52** (2008) 13. doi: https://doi.org/10.1016/j.molcatb.2007.10.011

4. *Gattuso, G., Barreca, D., Gargiulli, C., Leuzzi, U., Caristi, C.,* Flavonoid composition of citrus juices, Molecules **12** (2007) 1641. doi: https://doi.org/10.3390/12081641

5. *Puri, M., Kalra, S.,* Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids, World J. Microbiol. Biotechnol. **21** (2005) 753.
doi: https://doi.org/10.1007/S11274-004-5488-7

6. *Puri, M., Banerjee, U. C.,* Production, purification, and characterization of the debittering enzyme naringinase, Biotechnol. Adv. **18** (2000) 207.
doi: https://doi.org/10.1016/S0734-9750(00)00034-3

7. *Chang, H. Y., Lee, Y. B., Bae, H. A.,* Purification and characterisation of *Aspergillus sojae* naringinase: The production of prunin exhibiting markedly enhanced solubility with in vitro inhibition of HMG-CoA reductase, Food Chem. **124** (2011) 234.
doi: https://doi.org/10.1016/j.foodchem.2010.06.024

8. *Bodakowska-Boczniewicz, J., Garncarek, Z.,* Immobilization of naringinase from *Aspergillus niger* on a magnetic polysaccharide carrier, Molecules **25** (2020) 2731.
doi: https://doi.org/10.3390/MOLECULES25122731

9. *Mukund, P., Belur, P. D., Saidutta, M. B.,* Production of naringinase from a new soil isolate, *Bacillus methylotrophicus*: Isolation, optimization and scale-up studies, Prep. Biochem. Biotechnol. **44** (2014) 146.
doi: https://doi.org/10.1080/10826068.2013.797910

10. *Ribeiro, M. H.,* Naringinases: Occurrence, characteristics, and applications, Appl. Microbiol. Biotechnol. **90** (2011) 1883.
doi: https://doi.org/10.1007/s00253-011-3176-8

11. *Ni, H., Li, L., Xiao, A., Cao, Y., Chen, Y., Cai, H.,* Identification and characterization of a new naringinase-producing strain, *Williopsis californica* Jmudeb007, World J. Microbiol. Biotechnol. **27** (2011) 2857.
doi: https://doi.org/10.1007/s11274-011-0766-7

12. *Ni, H., Cai, H., Xiao, A., Chen, F., You, Q., Wang, Y.,* Improved purification of α-L-rhamnosidase from *Aspergillus niger* naringinase, World J. Microbiol. Biotechnol. **27** (2011) 2539.
doi: https://doi.org/10.1007/S11274-011-0723-5/METRICS

13. *Puri, M., Kaur, A., Barrow, C. J., Singh, R. S.,* Citrus peel influences the production of an extracellular naringinase by *Staphylococcus xylosus* MAK2 in a stirred tank reactor, Appl. Microbiol. Biotechnol. **89** (2011) 715.
doi: https://doi.org/10.1007/s00253-010-2897-4

14. *Pavithra, M., Prasanna, D. B., Saidutta, M. B.,* Production of naringinase by a new soil isolate of *Serratia* sp.: Effect of different carbon and nitrogen sources, Res. J. Chem. Environ. (2013).

15. *Puri, M., Kaur, A., Singh, R. S., Singh, A.,* Response surface optimization of medium components for naringinase production from *Staphylococcus xylosus* MAK2, Appl. Biochem. Biotechnol. **162** (2010) 181.
doi: https://doi.org/10.1007/s12010-009-8765-y

16. *Pegu, B. K.,* Isolation and molecular characterization of *Bacillus cereus*, a new naringinase producer, Int. J. Adv. Inn. Res. **8** (2019) 2278.

17. *Kumar, A., Singh, M. K., Amena, S.,* Optimization of naringinase production and its purification from *Micrococcus* sp., Int. J. Pharm. Pharm Sci. **7** (2015) 269.

18. *Phukan, K., Kardong, D.,* Isolation of naringinase producing soil bacteria from *Psidium guajava* L. and *Terminalia chebula* Retz and its enzymatic activity, AIMS Mol. Sci. **7** (2020) 292.
doi: https://doi.org/10.3934/molsci.2020014

19. *Radhakrishnan, I., Sampath, S., Kumar, S.,* Isolation and characterization of enzyme naringinase from *Aspergillus flavus*, Int. J. Adv. Biotechnol. Res. **4** (2013) 208.

20. *Davis, W. B.,* Determination of flavanones in citrus fruits, Anal. Chem. **19** (1947) 476.
doi: https://doi.org/10.1021/ac60007a016

21. *Oshadie De Silva, G., Marapana, R., Manawaduge, R.,* Effect of naringinase enzymatic treatment on the bitter compound naringin in fresh juice of "Bibila sweet" oranges, J. Pharmacogn. Phytochem. **6** (2017) 174.

22. *Bergey, D. H.,* Bergey's Manual of Determinative Bacteriology, 1994.

23. *Zhu, Y., Jia, H., Xi, M., Xu, L., Wu, S., Li, X.,* Purification and characterization of a naringinase from a newly isolated strain of *Bacillus amyloliquefaciens* 11568 suitable for the transformation of flavonoids, Food Chem. **214** (2017) 39.
doi: https://doi.org/10.1016/j.foodchem.2016.06.108

24. *Laemmll, U. K., Mölbert, E., Showe, M., Kellenberger, E.,* Form-determining function of the genes required for the assembly of the head of bacteriophage T4, J. Mol. Biol. **49** (1970) 99.
doi: https://doi.org/10.1016/0022-2836(70)90379-7

25. *González-Vázquez, R., Azaola-Espinosa, A., Osorio-Revilla, G.,* The effect of different carbon sources and salts in the production of naringinase by *Aspegillus niger* ATCC1015, Revista Mexicana de Ingeniera Quimica **10** (2011) 1.

26. *Chen, D. X., Niu, T. G., Cai, H. N.,* Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316, African J. Biotechnol. **9** (2010) 4970.
doi: https://doi.org/10.5897/AJB10.087

27. *Puri, M., Banerjee, A., Banerjee, U. C.,* Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344, Process Biochem. **40** (2005) 195.
doi: https://doi.org/10.1016/J.PROCBIO.2003.12.009

28. *VinothKumar, V., RevathiBabu, P. K. S.,* Optimization of fermentation parameters for enhanced production of naringinase by soil isolate *Aspergillus niger* VB07, Food Sci. Biotechnol. **19** (2010) 827.
doi: https://doi.org/10.1007/s10068-010-0116-9

29. *Kumar, V. V.,* Comparative studies on inducers in the production of naringinase from *Aspergillus niger* MTCC 1344, Afr. J. Biotechnol. **9** (2010) 7683.
doi: https://doi.org/10.4314/ajb.v9i45

30. *Igbonekwu, A., Omeje, K., Ezugwu, A., Eze, S., Njoku, O., Chilaka, F.,* Characterization of nariginase obtained from *Aspergillus niger* by submerged fermentation using naringin extracted from lemon peels, Res. Dev. Material. Sci. **4** (2018) 4.
doi: https://doi.org/10.31031/rdms.2018.04.000599

31. *Norouzian, D., Hosseinzadeh, A., Inanlou, D. N., Moazami, N.,* Production and partial purification of naringinase by *Penicillium decumbens* PTCC 5248, World J. Microbiol. Biotechnol. **16** (2000) 471.
doi: https://doi.org/10.1023/A:1008962131271

32. *Ni, H., Chen, F., Cai, H., Xiao, A., You, Q., Lu, Y.,* Characterization and preparation of *Aspergillus niger* naringinase for debittering citrus juice, J. Food Sci. **77** (2012) 1.
doi: https://doi.org/10.1111/j.1750-3841.2011.02471.x

33. *Zhu, Y., Jia, H., Xi, M., Li, J., Yang, L., Li, X.,* Characterization of a naringinase from *Aspergillus oryzae* 11250 and its application in the debitterization of orange juice, Proc. Biochem. **62** (2017) 114.
doi: https://doi.org/10.1016/J.PROCBIO.2017.07.012

34. *Puri, M., Marwaha, S. S., Kothari, R. M.,* Studies on the applicability of alginate-entrapped naringinase for the debittering of kinnow juice, Enzyme Microb. Technol. **18** (1996) 281.
doi: https://doi.org/10.1016/0141-0229(95)00100-X

35. *Puri, M., Seth, M., Marwaha, S. S., Kothari, R. M.,* Debittering of kinnow mandarin juice by covalently bound naringinase on hen egg white, Food Biotechnol. **15** (2001) 15 13.
doi: https://doi.org/10.1081/FBT-100103891