Advances in Bioresearch
Adv. Biores., Vol 16 (1) January 2025: 230-236
©2025 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.16.1.230236

Advances in Bioresearch

ORIGINAL ARTICLE

Purification, Characterization and Nano-Encapsulation of Naringinase for A Biotransformation of Flavonoid from *Aspergillus flavus* Kla-80

Ananda Sindhe, K.Lingappa*
[1]Department of Microbiology, Gulbarga University, Kalaburagi-585106.
***Corresponding Author:** lingappak@redifmail.com
(ORCID-0000-0001-7865-1442)
*(ORCID-0000-0002-5350-6422)

ABSTRACT
Naringinase is a multi-enzyme complex having α-L-rhamnosidase and β-D-glucosidase activity as it breaks down naringin into simple forms rhamnose and purnin which was further hydrolyzed to glucose and naringenin. In the present study, Naringinase isolated from Aspergillus flavus KLA-80 which was showed an activity of 867U/mL was selected for purification. The purified enzyme showed a 78.43% yield through a series of steps. The molecular weight of naringinase by using chromatography on a Sephadex G-200 column and molecular mass of subunit was estimated to be around 20Kd by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme was found to have maximum activity at temperature of 50^{0}C and found to stable at 45^{0}C to 55^{0}C. The optimum pH was found to be around pH 4 and the activity was found to be stable at 3-6 pH respectively. Further immobilization of purified enzyme was carried out by Nano-encapsulation method using homogenization technique where 37.9% of immobilization is achieved and also SEM analysis of naringinase was performed.
Keywords: -*Naringin, Naringinase, Citrus fruit, Immobilization, Bio-transformation.*

Received 10.10.2024 Revised 01.11.2024 Accepted 19.11.2024

How to cite this article:
Ananda Sindhe, K.Lingappa. Purification, Characterization and Nano-Encapsulation of Naringinase for A Biotransformation of Flavonoid from *Aspergillus flavus* Kla-80. Adv. Biores., Vol 16 (1) January 2025: 230-236.

INTRODUCTION

Nowadays, people are very much conscious about their dietary habits, due to which food industries are trying to make their products as healthy as possible without making any changes in their original food or fruits value in order to increase the trading of their product. As we know that fruits are rich with enormous variety of nutrients and bioactive compounds (1). There are various types of fruits present among which citrus fruit variety are rich in polyphenols content, specifically the flavanone, hesperidin's, naringin and narirutin along with Vitamin C, folate, potassium and pro-vitamin A (2) which provide prevention towards chronic type of diseases (3). Orange and grapefruit juices are naturally rich source of micro-nutrients and bioactive phytochemicals, particularly polyphenols which are associated with variety of nutritional, dietary and health benefits (4), as it helps in providing natural balance and combination of water, vitamins, sugar and acids. Controlled randomized procession showed a significant improvement in vascular function and blood pressure, followed by the consumption of 100% orange juice (OJ) with effects which is potentially mediated through hesperidin and potassium (5). Inspite of having these advantages the consumption of citrus juices is less because of the bitterness caused by the naringin. In order to overcome this microbial extraction of enzymes was done where enzymes are isolated from various bacteria and fungi sources (6). Whereas it is found that Naringinase has the ability to cleave naringin as it express α-L-rhamnosidase and β-D-glucosidase activity, which breaks the terminal end of α-L-rhamnose and β-D glucose from glycosides, glycopeptides, glycolipids and flavonoids (7). Naringinase which have the ability to cleave naringin makes it more attractive for the use in food industry in particular for di-bettering of fruit juices. Other than the food industry the enzyme also has an industrial application as it

plays an important role in biotransformation of steroids and antibiotics (8). Moreover, its hydrolyzed product can be used as starting material for the synthesis of substances applied in pharmaceuticals, cosmetics and food technology (9). In the present investigation, we divulge the production and characterization of partially purified Naringinase from *Aspergillus flavus* KLA-80 and also immobilization and SEM analysis of the purified naringinase was performed in order to debitter grapefruit juice.

MATERIAL AND METHODS

Organism: -

The previously isolated *Aspergillus flavus* (KLA-80) which was isolated from citrus growing regions, which was maintained in lab conditions in Naringin Agar Media (NAM), it was sub-cultured for every 5-7 weeks. Production and assay of Naringin by HPLC was reported earlier (10).

Cultivation of *Aspergillus flavus* on the media slant to prepare spore suspension: -

The strain KLA-80 was found able to synthesize Naringinase which was inoculated onto media slant with composition (g/l) of Peptone 1.5g, Naringin 1g, galactose 12.5gm, kcl 0.5gm, MgSo4 0.5gm, KH_2PO_4 1gm, $Fecl_3$ 0.1g. The spores were grown at PH 4.5 and at a temperature of 35^0C for 3 days before they were washed and adjusted spore suspension of $1x10^6$.

Enzyme Assay: -

Naringinase assay was carried out by the imperceptible modification of Thammawat method (11) using naringin as substrate (0.65 mL of 0.1%) in 0.01M acetate buffer (pH 4.0) was made to react with 0.1mL of Naringinase enzyme for 15 min at 60^0C. In the reaction mixture 0.2Ml was taken and mixed with 4mL of 90% diethylene glycol and 0.2Ml of 4N NaOH. The mixture was kept for 10 min at an ambient temperature. This resulted yellow color was measured at 420nm. Determination of the enzyme activity was calculated using pure naringin as standard. One unit (U) of Naringinase activity was defined as the amount of enzyme that could hydrolyze 1 µl/mol of naringin/mL in min at the assay conditions.

Protein Concentration: -

The concentration of protein was determined by measuring the absorbance at 280nm of the Naringinase solution by Lowry's method (12).

Enzyme Purification: -

The Naringinase enzyme was purified partially by ammonium sulfate precipitation method (13) followed later by dialysis. The fermented broth was precipitated using 65% saturation of ammonium sulfate and kept at 4^0C for overnight. The precipitated protein was centrifuged at 9000rpm for 5min. The resulting pellet was dissolved in 0.1M sodium acetate buffer of pH 4 and it was dialyzed against phosphate buffer (0.05M, pH 7.0) for 24hrs at 25^0C with timely replacement of buffer. The concentrated sample of 1ml was applied to anion exchange column Sephadex G-200 column (1.5-45cm) that had been previously equilibrated with 0.05 M phosphate buffer (pH 7) containing 0.15 M sodium chloride. Elution was performed at a flow rate of 0.5 ml min)1 (0.05 M phosphate buffer). Fractions (1 ml) corresponding to Naringinase activity were collected and checked for the purity of enzyme using native SDS-PAGE.

Characterization of Purified enzyme: -

Characterization of purified enzyme is done to determine the optimum pH and temperature of Naringinase, assay were carried out in different pH values using sodium acetate (0.05M,pH 3-6) and at different temperature (20-70^0C) and enzyme activity was assayed. Apparently, Km and Vmax values for naringin was calculated using a Line weaver-Burk plot method.

SDS polyacrylamide gel Electrophoresis and Molecular Weight Measuring: -

Naringinase molecular weight is estimated by SDS-PAGE and Gel filtration chromatography. The GFC was performed by Sephadex G-200 column as mentioned and SDS-PAGE was carried out.

Nano-encapsulation: -

To increase the enzyme efficiency, immobilization of naringinase was done by nano-encapsulation method as described by Donsi et al.,2011 (14). In which 2% of acetic acid or distill water dissolved in the polymer of sodium alginate and chitosan and rotated at 250rpm for about 48hrs by adjusting pH to 5.5 by using 0.5M sodium hydroxide solution to get a polymer at a concentration of 2%(w/v), followed by addition of 1g of nano-materials with 2ml of naringinase solution and kept at 4^0C for 24hrs until a desired naringinase load is obtained.

After the end of incubation period, the obtained materials were washed twice with 0.1M sodium acetate buffer, pH 4.0. The supernatant was separated and resuspended in deionized water until the naringinase assay is carried out.

Immobilization Assay: -

The assay of Nano-encapsulation of naringinase was carried out by the method prescribed by Khaled (15). In this method about 1g of previously immobilized naringinase was taken to which 2ml of 0.2%

naringin solution dissolved in 0.1M sodium acetate buffer pH 4.0. The amount of liberated glucose is estimated as stated earlier. The immobilization yield was calculated by the following equation:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme} \times 100}{\text{Enzyme added} - \text{Unbound Enzyme}}$$

SEM Analysis: -

The structure of the immobilized naringinase was observed by using scanning electron microscopy field emission gun (TESCAN-VEGA3 LMU) where the surface of the samples was analyzed by operating at 30kv.

RESULTS

Protein Assay

For protein purification, the fermented flasks were kept in an ice bath, the cell free extracts were recovered by the addition of 5-10ml of sodium acetate 50mM at pH 4 and further mixed using glass rod, eventually, the suspension was filtered and centrifuged at 9000rpm for 15min at 4°C, a clear brown supernatant was used for the subsequent analysis. Further, protein content was estimated using Lowry's method using bovine serum albumin as a standard. About 100ul of sample was taken for analysis. In which 65% of ammonium sulfate showed a highest protein concentration which was used for further.

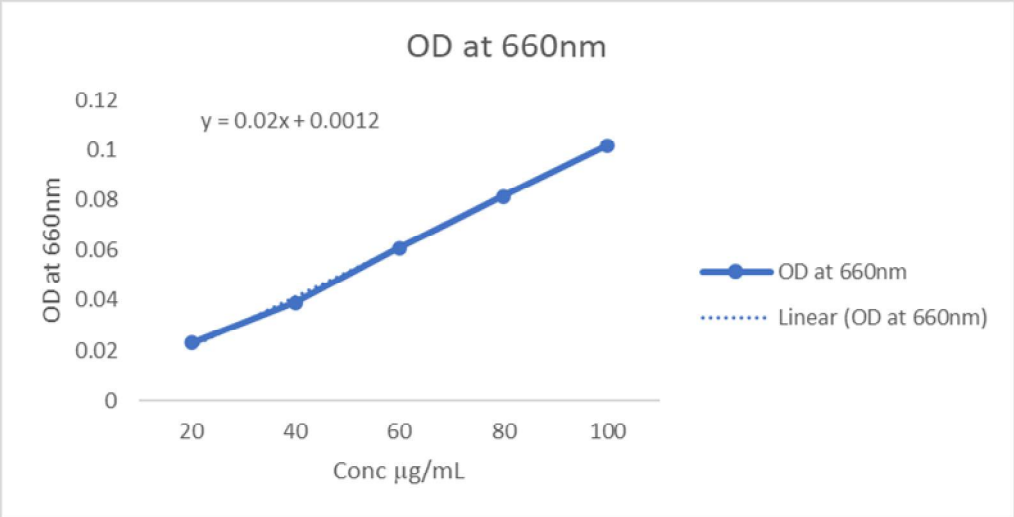


Fig 1: - Graph showing protein concentration

Table 1: -Protein concentration at different Ammonium sulfate concentration

Sl. No	Sample Description Ammonium sulphate precipitate % saturation	OD at 660nm	Protein content mg/Ml
1	50	0.139	68.9
2	55	0.195	96.9
3	60	0.301	149.9
4	65	0.440	219.4
5	70	0.150	74.4

Purification of Naringinase: -

A typical purification of naringinase from *Aspergillus flavus* KLA-80 was done by ammonium sulfate precipitate as explained above. As this step yields specific activity of 60.0 of the purified sample whereas crude enzyme showed a specific activity of 32.6 i.e. there is an increase in around 14.33 percent of specific activity was found. The ammonium sulphate was dialyzed using phosphate buffer (pH 7.0, 0.05M) overnight at 6°C. The dialyzed precipitation was lyophilized and dissolved in minimum quantity of phosphate buffer. The protein was further purified by DEAE cellulose column and naringinase activity was eluted at 0.45M NaCl.

Table 2: -Naringinase Purification Profile

Sl.No	Sample Description	Protein	Total Enzyme activity	Specific activity	Percentage yield
1	Crude extract	115.9	7650	32.6	100
2	Dialysed enzyme 4 pH 50°C	219.4	6750	36.6	88.23
3	Purified enzyme	250.8	6000	60.0	78.43

Characterization of Enzyme: -

The SDS-PAGE of different purified extract of naringinase was determined in below given figure with reference to a broad range of marker, by evidence that there were different subunits having molecular weight (MW). The molecular mass of the purified enzyme was 10-20, 60-65 and KDa, the purified naringinase gave 1 band with an apparent molecular mass of 20KDa respectively (As shown in below figure). The proteins were stained with Coomassie brilliant blue R-250.

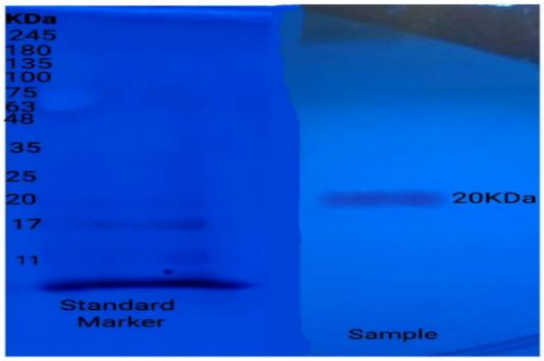


Fig2: - SDS-PAGE of Purified Naringinase

Effect of pH: -

The optimum pH of the purified enzyme was determined by dissolving the 0.1% naringin at various sodium acetate buffer concentrations varies from different pH from 3.0 to 8.0 in which optimum pH was found to be around 4.0 though at pH 5 and 6 it is relatively active having lost its activity. The broad pH optimum is preferable for application of naringinase in food and pharmaceutical industry. This pH optimum under the condition used for similar to those reported for naringinase from other micro-organisms. The enzyme optimum Ph of the enzyme at Ph 4.0 of *Aspergillus niger* 1344 (13) whereas *Pencillium decumbens* showed an optimum Ph of the enzyme 4.0 (16) *Clostridium stercorarium* was found to showed highest activity in the Ph 5.5(17).

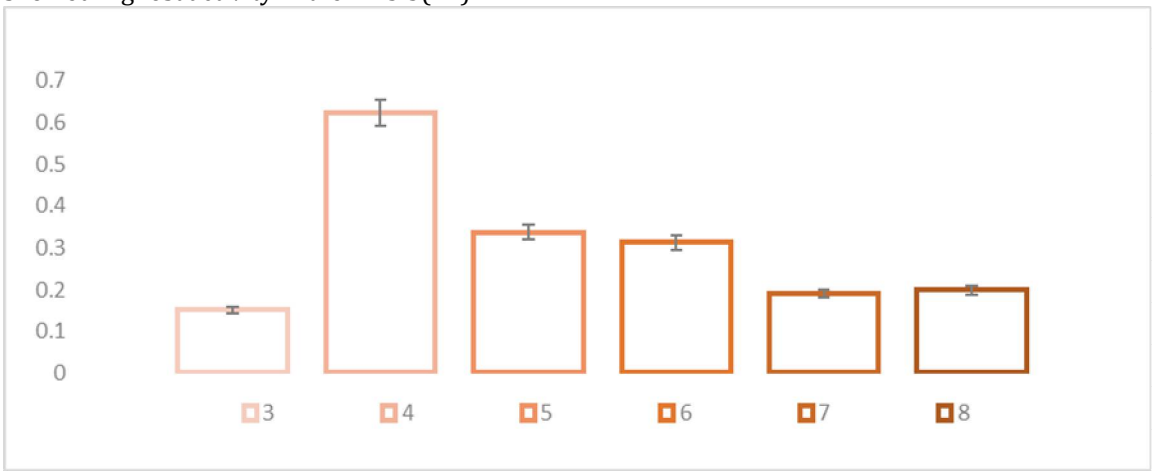


Fig3: Effect of Ph

Effect of Temperature: -

The temperature has an effect on enzyme activity i.e., the activity increases with increase in temperature and at some point causing activity to be lost by denaturation. The optimum temperature of the purified naringinase was found to be at 50^{o}C and completely decreases after 60^{o}C.As per the earlier studies of Kanokpan Thammawat *et al* [18] reported optimum temperature of 60^{o}C for purified naringinase from

Aspergillus niger BC 25166, whereas *Aspergillus Brasiliensis* MTCC 1344 showed highest activity at 60°C (18), Hui Nui *et al* (19) isolated *Aspergillus niger* which showed an optimum temperature in the range of 45 to 55°C where Manzanares et al 1997(20) reported optimum temperature of the 65 °C of the purified rhamnosidase isolated from *Aspergillus niger*.

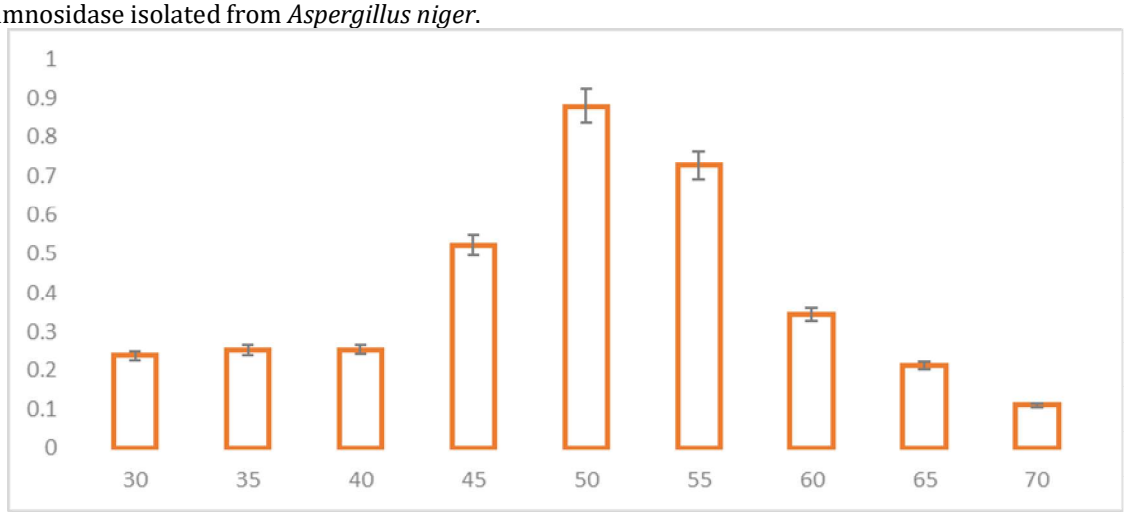


Fig4: Effect of Temperature

Immobilization of the enzyme: -

The Nano-encapsulation data was showed in below table 3 and Fig 2. Showed an increase in enzyme specificity. The yield of encapsulated naringinase obtained after Nano-encapsulation in sodium alginate beads and chitosan.

Sample	Enzyme Activity (IU)
Pure Enzyme	0.165
Immobilized Enzyme	0.044
Unbound Enzyme	0.049

Table3: - Enzyme Activity at different stages

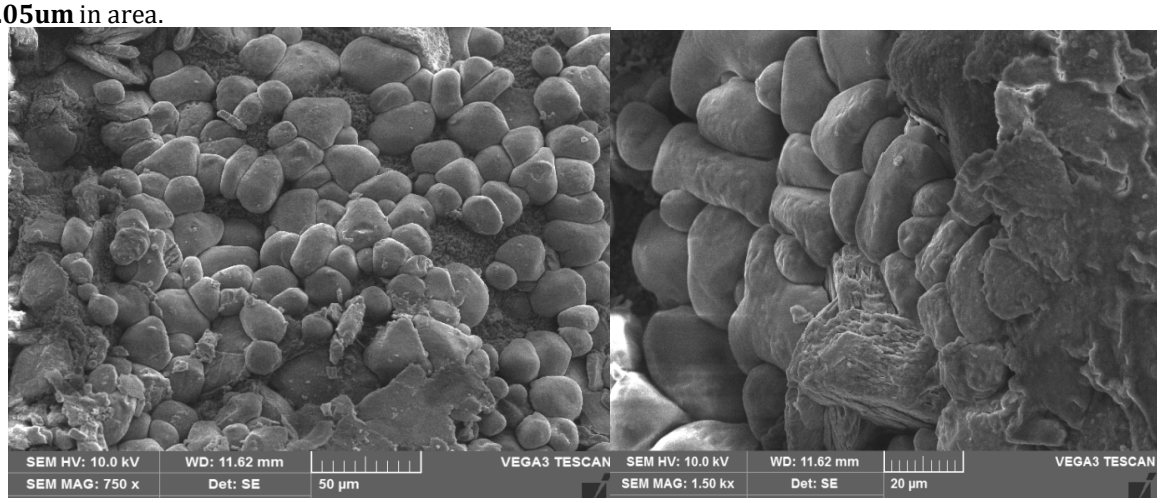
Fig5: - Immobilization of Naringinase

The enzyme molecule orients themselves to remain active blocked site, thereby restraining the active site of the enzyme towards the substrate. Longer the incubation time could increase the probability for covalent modification close to the active site increasing the enzyme inactivation where the 37.9% of immobilization was achieved, whereas silk fibroin which was used for the immobilization of naringinase where >70% residual activity was retained at an optimum reactive temperature at 55°C (19). Sol-gel developed naringinase immobilization was done for *Penicillium decumbens* increasing an immobilization yield up to 82% (21) other than these naringinase immobilized with calcium alginate beads showed 25% of retention in activity compared with initial maximum rate (22).

SEM Analysis: -

The surface morphology of the immobilized naringinase were examined by SEM in which the size of the cell was measured with 50µm at a magnum of 750x and 10 µm at a magnum of 5.00kx and the size of the Nano-particle was found to be 2 µm in a SEM magnum of 20.0kx. After pre-treatment and dehydration of

sample was mounted with gold sputtering. Different resolutions from 500-2000um were to study the changes in microscopic structures and particles. The SEM images shows the average size of cell is around **3.05um** in area.



a b

Fig6(a &b): - SEM micrographs of the Naringinase enzyme a& b encapsulated with sodium alginate and chitosan

CONCLUSION

Bitterness of citrus fruit has been a one of the major drawbacks of industries in regard to this naringinase has gained importance in industrial application. Isolation and production of naringinase by micro-organisms is much cheaper and more efficient than chemical synthesis and extraction**.** In the present study, we have successfully isolated naringinase enzyme using *A. flavus* KLA-80 further. The optimum pH and incubation temperature of naringin hydrolysis was found to be at 4 and 50^0C, respectively. Further by immobilizing the enzyme with Nano-encapsulation achieving around 37.9% of immobilization along with SEM analysis was also carried out for the determination of enzyme size.

ACKNOWLEDGEMENT

The author is very much thankful to the Karnataka Fund for Infrastructure Strengthening in Science and Technology in Higher Educational Institutions (K-FIST) and Karnataka Science and Technology Promotion Society (KSTePS), Department of Science and Technology, Karnataka, in helping to develop lab infrastructure and providing financial support to carry out this research work.

REFERENCES

1. Joana Gil-Chávez, G., Villa, J. A., Fernando Ayala-Zavala, J., Basilio Heredia, J., Sepulveda, D., Yahia, E. M., & González-Aguilar, G. A. (2013). Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. Comprehensive Reviews in Food Science and Food Safety, 12(1), 5–23. doi:10.11111/1541-4337.12005
2. Manach, C., Morand, C., Gil-Izquierdo, A., Bouteloup-Demange, C., & Rémésy, C. (2003). Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. European Journal of Clinical Nutrition, 57(2), 235–242. doi:10.1038/sj.ejcn.1601547
3. Sánchez Macarro, M., Martínez Rodríguez, J. P., Bernal Morell, E., Pérez-Piñero, S., Victoria-Montesinos, D., García-Muñoz, A. M., Cánovas García, F., Castillo Sánchez, J., & López-Román, F. J. (2020). Effect of a Combination of Citrus Flavones and Flavanones and Olive Polyphenols for the Reduction of Cardiovascular Disease Risk: An Exploratory Randomized, Double-Blind, Placebo-Controlled Study in Healthy Subjects. Nutrients, 12(5), 1475. doi:10.3390/nu12051475
4. Russo, C., Maugeri, A., Lombardo, G. E., Musumeci, L., Barreca, D., Rapisarda, A., Cirmi, S., & Navarra, M. (2021). The Second Life of Citrus Fruit Waste: A Valuable Source of Bioactive Compounds. Molecules, 26(19), 5991. doi:10.3390/molecules26195991
5. Dourado, G. K. Z. S., & Cesar, T. B. (2015). Investigation of cytokines, oxidative stress, metabolic, and inflammatory biomarkers after orange juice consumption by normal and overweight subjects. Food & Nutrition Research, 59(1), 28147. doi:10.3402/fnr.v59.28147
6. Purewal, S. S., & Sandhu, K. S. (2021). Debittering of citrus juice by different processing methods: A novel approach for food industry and agro-industrial sector. Scientia Horticulturae, 276, 109750. doi:10.1016/j.scienta.2020.109750

7. Nunes, M. A. P., Vila-Real, H., Fernandes, P. C. B., & Ribeiro, M. H. L. (2009). Immobilization of Naringinase in PVA–Alginate Matrix Using an Innovative Technique. Applied Biochemistry and Biotechnology, 160(7), 2129–2147. doi:10.1007/s12010-009-8733-6
8. Ribeiro, M. H. L., & Rabaça, M. (2011). Cross-Linked Enzyme Aggregates of Naringinase: Novel Biocatalysts for Naringin Hydrolysis. Enzyme Research, 2011, 1–8. doi:10.4061/2011/851272
9. Yadav, M., Sehrawat, N., K. Sharma, A., Kumar, V., & Kumar, A. (2018). Naringinase: Microbial Sources, Production and Applications in Food Processing Industry. Journal of Microbiology, Biotechnology and Food Sciences, 8(1), 717–720. doi:10.15414/jmbfs.2018.8.1.717-720
10. Sindhe, A., & Lingappa, K. (2023). Isolation and Molecular Characterization of the Naringinase Producing Micro-organisms for the Bio-transformation of Flavonoid. Journal of Pure and Applied Microbiology, 17(1), 456–466. doi:10.22207/jpam.17.1.38
11. 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. 42: 61-72 (2008).
12. Lowry, O.H., Rosebroug, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
13. Puri, M., & Kalra, S. (2005). Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. World Journal of Microbiology and Biotechnology, 21(5), 753–758. doi:10.1007/s11274-004-5488-7
14. Donsi, F.M., Annunziata, M., Sessa, M., and Ferrari, G. 2011. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. LWT-Food Science and Technology. 44, 1908–1914.
15. Khaled, F.M., Heba, I.A., and Manal, M.H. 2018. Micro- and nano capsulated fungal pectinase with outstanding capabilities of eliminating turbidity in freshly produced juice. Food Science and Technology International. 24, 330–340.
16. Nunes, M. A. P., Vila-Real, H., Fernandes, P. C. B., & Ribeiro, M. H. L. (2009). Immobilization of Naringinase in PVA–Alginate Matrix Using an Innovative Technique. Applied Biochemistry and Biotechnology, 160(7), 2129–2147. doi:10.1007/s12010-009-8733-6.
17. Puri, M., Kaur, A., Schwarz, W. H., Singh, S., & Kennedy, J. F. (2011). Molecular characterization and enzymatic hydrolysis of naringin extracted from kinnow peel waste. International Journal of Biological Macromolecules, 48(1), 58–62. doi:10.1016/j.ijbiomac.2010.09.012
18. Shanmugaprakash, M., Vinothkumar, V., Ragupathy, J., & Reddy, D. A. (2015). Biochemical characterization of three phase partitioned naringinase from Aspergillus brasiliensis MTCC 1344. International Journal of Biological Macromolecules, 80, 418–423. doi:10.1016/j.ijbiomac.2015.06.057
19. Ni, H., Chen, F., Cai, H., Xiao, A., You, Q., & Lu, Y. (2011). Characterization and Preparation of *Aspergillus niger* Naringinase for Debittering Citrus Juice. Journal of Food Science, 77(1). doi:10.1111/j.1750-3841.2011.02471.x
20. Manzanares, P., deGraff, L.H. & Visser, J. 1997 Purification and characterization of an a-L-rhamnosidase from *Aspergillus niger*. FEMS Microbiology Letters 15, 279–283
21. Vila-Real, H., Alfaia, A. J., Rosa, M. E., Calado, A. R., & Ribeiro, M. H. L. (2010). An innovative sol–gel naringinase bioencapsulation process for glycosides hydrolysis. Process Biochemistry, 45(6), 841–850. doi:10.1016/j.procbio.2010.02.004
22. Afonso, C., Ferreira, L., Vila-Real, H., Alfaia, A. J., & Ribeiro, M. H. L. (2007). High pressure and temperature combination with naringin hydrolysis by naringinase Ca-alginate beads in grapefruit juice processing: Bitterness and microbial reduction. Journal of Biotechnology, 131(2), S265. doi:10.1016/j.jbiotec.2007.07.483.

Copyright: © 2025 Author. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.