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Debittering citrus fruit juices, advances in immobilized enzyme technology: A review

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Abstract
The use of immobilised enzyme technology for debittering citrus fruit juices is covered in this review article. The three major parts of the essay are the isolation of naringinase, the immobilisation of naringenase, and the debittering of citrus fruit juice by immobilised enzyme. The substance that causes the initial bitterness in citrus fruit juice, naringin, is hydrolyzed by the enzyme naringinase. In order to employ enzymes in the debittering of citrus fruit juices, several methods at immobilising enzymes on a suitable support are discussed in this work. These include biotechnological methods using enzymes and physicochemical processes. Several methods have been used to immobilise naringinase, including trapping in natural polymer beads, elimination of between 70 and 95 percent of the naringin contained in various citrus juices, and covalent immobilisation in hetero-functional supports. The usage of immobilised naringinase for debittering grapefruit juice as well as the simultaneous hydrolysis of naringin and adsorption of limonin are also covered in this article. The method to manufacture non-bitter citrus juice using immobilised enzymes has not yet been commercialised internationally, despite the huge amount of citrus fruit production.

Keywords: Debittering, naringin, immobilization, biotransformation, naringinase

1. Introduction
The citrus genus is one of the most widely grown fruit crops in the world, with products utilised in both food processing and fresh juice production. Citrus is a Rutaceae family of plants that includes orange varieties, sweet and sour oranges, lemon, tangerines (mandarins), and tangors. Every species or hybrid cross has one or more variations [12]. Secondary metabolites such as polyphenols and terpenoids, as well as vitamins A, C, and E, vital minerals, and dietary fibres, are abundant in citrus fruits. Consumers utilise citrus fruits in raw form to make beverages such as lemonade or limeade by diluting the fresh juice with sugar solution, and citrus juice is also mixed with juices from other fruits [5]. Flavonoids are phenolic substances that exist naturally and have a wide spectrum of bioactivities. The basic flavonoid structure has 15 carbon atoms and three rings, two of which are benzene rings linked by a three-carbon chain. Citrus fruits contain a range of bioactive compounds in their peels, pulp, seeds, and juice. (Panche *et al*., 2016). Citrus-derived flavonoids have been examined for their possible health benefits for over 30 years. There are 6 types of flavonoids. Flavones, Flavan-3-ols, Flavanones, Anthocyanins and Iso-flavones [23]. The bitterness in the citrus juice is mainly due to the presence of Naringin. Naringin (5,7-trihydroxyflavonone-7-rhamnoglucoside) is a flavanone glycoside found in grapes and citrus fruits [6]. Citrus juice is used on a regular basis thus enterprises are working on developing technology that can be used to preserve it for a long time. However, the bitterness of citrus juice is the major barrier to its processing. Due to the release of certain compounds during the extraction process, the bitterness of citrus fruits rises throughout processing hours, and this type of bitterness is known as 'delayed bitterness', The processing of citrus fruit juice has faced formidable problems in terms of bitterness and delayed onset of bitterness [26]. Naringinase is a debittering enzyme used in the industrial manufacture of citrus juices. It breaks down the chemical Naringin, which is responsible for the bitter taste of citrus liquids. It's a multi-enzyme complex with active centres for Alpha-L-rhamnosidase and beta glucosidase [20]. The presence of Naringin in citrus juice is primarily responsible for its bitterness (naringenin-7-Oglucorhamnoside). As a result, bitterness is eliminated when Naringin is converted to its aglycone moiety [18]. In contrast to other debittering procedures, enzymatic conversion utilising naringinase is recommended since it does not interfere with the organoleptic features or nutritional contents of the fruit juices.

Naringinase is a two-subunit complex that functions as a rhamnosidase and glucosidase. Rhamnosidase converts Naringin to prunin (4'-5,7-trihydroxyflavanone-7-glucoside) with the release of rhamnose first, followed by glucosidase hydrolyzing prunin to naringenin (4,5,7-trihydroxyflavanone) with the release of glucose. Prunin can inhibit protein tyrosine phosphatase 1B (PTP1B) and increase glucose absorption in insulin-resistant HepG2 cells, making it anti-diabetic and anti-bacterial. Naringenin is the aglycone derivative of prunin [10]. However, the polarity and solubility of naringin, prunin, and naringenin varies significantly. Naringenin, for example, is practically water insoluble, whereas Naringin and Prunin may be dissolved in water. These discrepancies may cause problems with simultaneous extraction, resulting in poor analytical findings. Different studies from across the world have reported a variety of microbiological sources of naringinase. Most research concentrate on fungus as a source [4]. There are also a few reports on bacterial synthesis of naringinase. *Aspergillus sp.* and *Penicillium sp.* strains have been shown to contain naringinase activity in particular among a variety of microbial sources. A significant source of naringinase has been identified as *Aspergillus niger*. Naringinase has been utilised to enhance grapefruit juice's antioxidant activity in addition to its usage in the debitterization of juices. It also has a preventive function against oxidative stress in grapefruit juice [16]. High selectivity biological catalysts are enzymes. The majority of the time, cofactors or prosthetic groups are combined with a protein component (biopolymer) to form an enzyme. The chemical process that leads to equilibrium is sped up by these biological catalysts, without causing it to shift its location or go through any minor chemical changes. An enzyme can be said to be immobilised when it is restricted to a phase (matrix or support) other than the substrates and products. Typically, carrier matrices consist of inert polymers and inorganic substances [4]. Immobilised enzymes are described as enzymes that are physically contained or localised in a certain specified region of space while maintaining their catalytic functions. A variety of techniques, ranging from reversible physical adsorption and ionic connections to stable covalent bonding, can be utilised to immobilise an enzyme. These approaches can be categorised in a variety of ways. In one way, there are two main categories for immobilisation techniques: reversible and irreversible techniques. In addition to this classification, immobilisation techniques such as support binding and entrapment techniques have also been separated based on the kind of chemical reaction employed to bind [14]. Naringin and naringenin hydrolyze one another in a chemical process. Naringenin may be produced by hydrolyzing naringin with acid. The aglycone of naringin is called naringenin, and it is naringin without the sugar molecule connected to it. Naringenin is created as a result of the hydrolysis process, which destroys the connection between the sugar molecule and naringin. Naringin can be hydrolyzed with the immobilised naringinase in a more effective and stable way to yield naringenin [27]. To ascertain its reusability, the operational stability of immobilised naringinase can be examined. Through the use of equilibrium dialysis and liquid chromatography with electrospray ionisation tandem mass/mass spectrometry, the protein binding ratios of naringin and naringenin in rat, dog, and human plasma have been examined [18].

2. Isolation of naringinase

Soil bacteria and fungus like Aspergillus niger and Aspergillus flavus are only a few of the sources from which naringinase may be extracted. Following the naringinase's breakdown of the substrate is how the isolation procedure determines the activity of the enzyme. It has also been researched how to characterise and purify naringinase. Use of response surface approach can improve the naringin extraction procedure. One research went into detail on the isolation and characterization of the Aspergillus flavus naringinase enzyme. Another study's goal was to find a mould with strong naringinase production capabilities, high -L-rhamnosidase activity, and effective catalysis at low pH [25]. Srikantha *et al*. (2016) isolated a naringinase-producing fungus from citrus fruit in order to flavour the juice and characterise the fungus. Aspergillus flavus had the highest naringenase activity (1.92 mol/ml/min) when all naringinase-producing fungi were treated to liquid fermentation medium for eight days at room temperature and 200 rpm. The Aspergillus flavus naringinase activity began on the second day, but the greatest activity (449.58Ug-1Dry Matter) was only discovered on the eighth day. Large-scale naringinase enzyme synthesis employing solid state fermentation and rice husk as a support is possible [26]. Puriwal *et al*., (2012) [19] isolated and tested Four strains of Naringin degrading bacteria for naringinase activity. Extracellular naringinase activity was seen in all four samples. Serratia Sp. was discovered phenotypically from the one that consistently had strong activity in three different medium (2 U/L). Different carbon and nitrogen sources' effects were investigated in shake-flask tests. The synthesis of naringinase was improved by glucose the most among all the carbon sources. Ammonium nitrate-supplemented peptone was shown to be advantageous. The medium containing naringin, glucose, peptone, ammonium nitrate, and salts produced a maximum of 9.2 U/L naringinase activity [21]. Panche *et al*., (2021) [15] isolated naringinase enzyme from Citrus macroptera peel and pomace using Aspergillus niger for immobilization and debittering of juice. On the physicochemical and phytochemical features of the juice, a comparison study of the effects of debittering employing immobilised enzyme and resin was conducted. With a regression value of 0.92, the adsorption of naringin onto resin followed pseudo-first order. The enzyme lowered the naringin concentration by 79.76% in 120 minutes and the resin by up to 73.28% in 4 minutes. Juice's significant bioactive components could be kept after treatments lasting less than 2 minutes and 90-120 minutes with resin and enzyme, respectively. Immobilisation also preserved 62% of the enzyme's activity after 2 months at 4 °C [26] Phukan and Kardongfour (2020) researched on strains of naringin degrading bacteria that were isolated from the soil of *Psidium guajava* L. and *Terminalia chebula* were investigated for naringinase activity of soil microbes and their growth conditions at different parameters. All four isolates demonstrated strong extracellular naringinase activity. Rod and oval-shaped gramme positive bacteria among the four isolates had the lowest specific activity (231.77 U/mg), whereas rod-shaped gramme negative bacteria displayed the greatest specific activity (405.31 U/mg). Of the four isolates, the rod-shaped isolate had the highest protein content and maximal growth. These findings indicated that the isolates also generated certain additional proteins in addition to the naringinase enzyme. These proteins may be important for

promoting the development of the bacteria [26]. It was discovered that extracellular naringinase activity was present in five isolated strains of naringin-degrading bacteria. The selected medium was where Bacillus methylotrophicus was most active. By using two-level complete factorial analysis with 24 shake flask tests, the optimal mix of carbon-nitrogen sources was found. Compared to the baseline medium, naringinase activity in the sucrose-yeast extract significantly increased (7.46 U/L). At a pH of 6 for the starting medium, the highest naringinase activity of 8 U/L was seen. Based on a central composite design, response surface modelling was used to ascertain the impacts of three independent variables (sucrose, yeast extract, and naringin), as well as how they interacted with one another. Studies in bioreactors using the improved medium revealed that after 34 hours of fermentation, 12.05 U/L of enzyme were produced [2]. zhu *et al*., (2017) [27] isolated naringinase from Bacillus amyloliquefaciens 11568 from soil was purified, identified, and characterized. The purified enzyme was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) examination, which revealed a single protein band with a molecular mass of 32 kDa. Below 45 °C and between pH 3.5 and 8.5, the enzymes were stable. The isolated naringinase's Km and Vmax were 0.95 mmol/L and 3847.3 mmol/(Lmin), respectively. The isolated naringinase had the power to break down additional glycosides like neohesperidin and naringin. In order to get rid of the naringin and lessen the bitterness of the juice, 4 U/mL of the enzyme was enough to be added to the citrus juice. These findings shed extensive light on the naringinase's structure and the hydrolysis of naringin [8]. Aspergillus niger emerged as the most promising of fifteen marine-derived fungus that had been locally isolated from Ismalia, Egypt, for the synthesis of naringinase enzyme. Orange rind was employed as a substrate containing naringin in solid state fermentation (SSF) of the agricultural industry waste. Using a Plackett-Burman factorial design, the effects of 19 factors were evaluated for their relevance on the synthesis of naringinase. The best combinations of the main media components for maximum naringinase production were assessed using statistical analysis (ANOVA). They are as follows: 15 g of discarded orange peel, 30 mL of moisture, 1% grape fruit, 1% NaNO3, 0.5% KH2PO4, 5 mM MgSO4, 5 mM FeSO4, and a starting pH of 7.5. The activity achieved was 3.14 times more than the basal production medium [1].

3. Immobilization of naringinase

Isolating naringinase can be done in a number of ways. Using silica materials with various pore sizes, a technique includes immobilising naringinase. The most naringinase activity has been seen in it. Naringin, naringinin, and other components are used in the production of naringinase by Aspergillus niger KMS. Using a new thermally stable biopolymer for the hydrolysis of naringin, covalent immobilisation of microbial naringinase. The immobilised naringinase has demonstrated great operating stability, a wide pH application range, and strong heat stability. The culture medium must be optimised, the enzyme must be immobilised, and the support for immobilisation of the enzyme must be characterised. These techniques support the use of naringinase in commercial fruit juice processing by providing an appropriate carrier material and some fundamental knowledge [10]. Sachdeva *et al*., (2014) [22] did a research to increased catalytic activity of naringinase, where it was was covalently bound to woodchips. The immobilization of naringinase on glutaraldehyde-coated woodchips was enhanced using 1% glutaraldehyde cross-linking. When compared to the soluble enzyme, the pH–activity curve of the immobilized enzyme changed toward a lower pH. The enzyme's heat stability improved dramatically when it was immobilized. The immobilized naringinase remained stable at 4 ºC throughout storage. When the immobilized enzyme was employed for seven consecutive cycles of operations, there was no loss of activity. Immobilization efficiency was 120%, whereas hydrolysis of normal Naringin by soluble naringinase was 82% efficient under ideal circumstances. Its use in debittering kinnow mandarin juice resulted in a 76% debittering efficiency. Naringinase was immobilized into a polymeric matrix consisting of poly (vinyl alcohol) (PVA) hydrogel, cryo-structured in liquid nitrogen, to create bio-catalytically active beads, which were induced from *Aspergillus niger* CECT 2088 cultures. Immobilization efficiency was investigated in relation to matrix content, enzyme load, and pH. Depending on the immobilization settings utilized, between 95% and 108% of the additional naringinase was actively entrapped in PVA cryogel. 8% (w/v) PVA at pH 7 and 1.6–3.7 U ml^{-1} enzyme load were the best conditions. When naringinase was immobilized, the pH/activity profiles showed no change in shape or optimal pH (4.5). However, immobilization moved the optimal temperature from 60 ˚C -70 ˚C and reduced the activation energy of the process, E_a, from 8.09 kJ $mol^{-1.}$ to 6.36 kJ $mol^{-1.}$ The entrapped naringinase may be reused six times (at 20˚C for 24 hours), preserving 36% effectiveness for naringin hydrolysis in simulated juice[2]. Patil *et al*., (2021) [17] researched on immobilization of naringinase in porous glass by means of different chemical methods concluded that for enzymatic activity to be demonstrated, both the chemical modification level and the microenvironment given by the support are required. Similarly, the support's pore size had a significant impact on metrics like enzyme load and kinetic constants. Taking this into account, as well as the lower Michaeli's constant and activation energy values, the lack of enzymatic activity losses during immobilization, and the high storage and operational stabilities, naringinase immobilized into CPG-460 via diazo coupling appears to be a suitable derivative for use in debittering citrus juices. Lei *et al*., (2011) [10] conducted a research where Naringinase was bound to mesoporous silica MCM-41 via adsorption with glutaraldehyde and used to debitter white grapefruit. Km value of the immobilized naringinase was lower than that of free naringinase. The immobilized catalysts showed excellent thermal stability and storage stability and could be recycled 6 times retained about 44.57% activities. The unaltered structural order of the prepared catalyst was characterized with reference to bulky and surface properties by infrared spectroscopy (FT-IR), elemental analysis and nitrogen adsorption–desorption isotherms analysis. Carceller, (2020) [3] conducted an research on immobilization of Naringinase from *Penicillium decumbens* on Chitosan Microspheres for Debittering Grapefruit Juice. The aim of this study was to immobilize naringinase on chitosan microspheres triggered with glutaraldehyde and then use the immobilized enzyme for grapefruit juice debittering. The effect of naringinase concentration on the immobilized enzyme's characterization in comparison to the soluble enzyme was examined. Both free and immobilized naringinase showed maximal activity at

optimal pH 4.0. Upon immobilization, however, the optimal temperature was lowered from 70 °C to 40 °C. The immobilized naringinase has a higher KM value than the soluble naringinase. The enzyme's thermal stability was unaffected by immobilization. The immobilized naringinase was stable in its function. After ten rounds of naringin hydrolysis from fresh grape fruit juice, this product preserved 88.1 ± 2.8% of its starting activity. The findings suggest that naringinase immobilized on chitosan might be used to improve the sensory characteristics of grape juices by debittering them. Muñoz *et al*., (2022) [13] used a combination biochemical and physical technique to analyses the decrease in both bitter components at the same time. The suggested technique relied on the employment of hetero-functional supports containing glyoxyl groups for covalent immobilization of naringinase, which hydrolyzes naringin, and alkyl groups for limonin adsorption. Butyl-glyoxyl agarose (BGA) and octyl-glyoxyl agarose (OGA) were used as supports, and they were characterized using aldehyde group quantification and FTIR analysis. The pH and temperature of free and immobilized enzymes were determined to be optimum. Supports' maximal enzyme loading capacity was investigated. Grapefruit juice debittering was tested using soluble enzyme, enzyme-free supports, and immobilized catalysts. The use of enzyme immobilized in BGA reduced naringin and limonin concentrations by 54% and 100%, respectively, while catalyst immobilized in OGA allowed for reductions of 74% and 76%, respectively, resulting in a final concentration of both bitter components below their detection threshold. When OGA biocatalyst was used instead of soluble enzyme or enzyme-free support, the results were improved. In five separate batches, biocatalyst was effectively used in juice debittering. Matrix screening for naringinase immobilization revealed that 2% sodium alginate was the best choice. 30 U of naringinase immobilized gave 82% naringin hydrolysis in 3 h, broadening of pH optima has attributed desirable flexibility for debittering kinnow juice of varying pHs, and temperature profiles indicated improved thermo-stability, which could be useful during the reduction in debittering costs. Alginate allowed for easy equilibrium with no obstruction in the inflow of naringin and the outflow of naringin or prunin, as well as good mechanical stability, indicating that it might be commercially exploited. Kinetic parameters designed with pure naringin were applied to kinnow juice and resulted in 60% debittering. It recommended ultrafiltration to reduce product inhibition and optimize naringin hydrolysis, as well as fortification with the permeate at the conclusion of debittering to create sweetened kinnow juice with healthy and natural qualities. Efforts are ongoing to transition from a batch to a continuous column process [21].

4. Debittering of citrus fruit juice by immobilized enzyme

One of the main issues with the citrus fruit juice industry and a substantial financial issue is citrus fruit bitterness. There are several physiochemical and enzymatic procedures that may be used to lower the bitterness in citrus juices below the level required for consumer acceptance. Due to its remarkable efficacy in eliminating bitter flavonoids, debittering of citrus fruit juice using immobilised enzyme technology has attracted interest in the fruit juice business. Through physicochemical and enzyme biotechnological methods, attempts have been made to employ immobilised enzymes on an adequate support to increase the consumer's acceptance and cost-effectiveness of fruit juice[19]. In order to hydrolyze naringin, recombinant α-L Rhamnosidase from a bacterial source was immobilised in calcium alginate beads using the entrapment technique. This method is economically appropriate for the debittering of fruit juices since the immobilised rhamnosidase was able to hydrolyze the naringin content in kinnow juice repeatedly. Aspergillus terreus' α-L-rhamnosidase has also been immobilised on a bagasse-particle-based matrix and was discovered to hydrolyze naringin contained in Citrus sinansis juice, demonstrating its usefulness in the debittering of citrus fruit liquids [24]. Ladole *et al*. (2021) [9] conducted a study where pectinase and naringinase enzymes were co-immobilized on environmentally friendly chitosan coated magnetic nanoparticles (chitosanMNPs) by cross-linking employing chitosan as a macro-molecular cross-linker. At a ratio of 1:3, 3% cross-linker concentration, and 150 min of cross-linking duration, both enzymes' maximal activity recovery in the co-immobilized state was achieved. The co-immobilized biocatalysts demonstrated a 52% reduction in turbidity and an 85% reduction in the naringin level when used to clarify and debitter grapefruit juice in a single pot. The co-immobilized enzymes were recycled up to the seventh cycle and are easily kept at ambient temperature for 30 days, keeping up to 64% and 86% residual activity, respectively [7]. An enzyme membrane reactor (EMR) is created in order to immobilise naringinase on a polyethersulfone ultrafiltration membrane utilising a fouling-induced method. The effects of membrane design, applied pressure, enzyme concentration, and pH are investigated in terms of permeate rate, immobilisation efficiency, and biocatalytic conversion. The intermediate pore blockage model was revealed to be the primary fouling mechanism for the enzymatic immobilisation. The EMR was employed to lessen the bitterness of grapefruit juice while maintaining its antioxidant properties. The immobilised enzyme was also kept at 4 °C for an extra night after each run of the method, where it maintained a high level of biocatalytic activity [25]. Carceller *et al*., (2020) [3] researched on Covalent immobilization of naringinase over two-dimensional 2D zeolites and its applications in a continuous process to produce citrus flavonoids and for debittering of juices. After glutaraldehyde was applied to the surface of the two-dimensional zeolite ITQ-2, the crude Penicillium decumbens naringinase and a refined naringinase with high -L-rhamnosidase activity were able to be covalently immobilised. With the aid of the substrate p-nitrophenyl-alpha-L-rhamnopyranoside (Rha-pNP), it was possible to examine the effects of pH and temperature on the enzyme activity (in free and immobilised forms) as well as the thermal stability. With a conversion rate > 90% and great selectivity, the naringin was hydrolyzed using the crude and purified naringinases supported on ITQ-2, resulting in the flavonoids naringenin and prunin, respectively [21]. Debittering of citrus fruit juice process is depected in Fig.1.

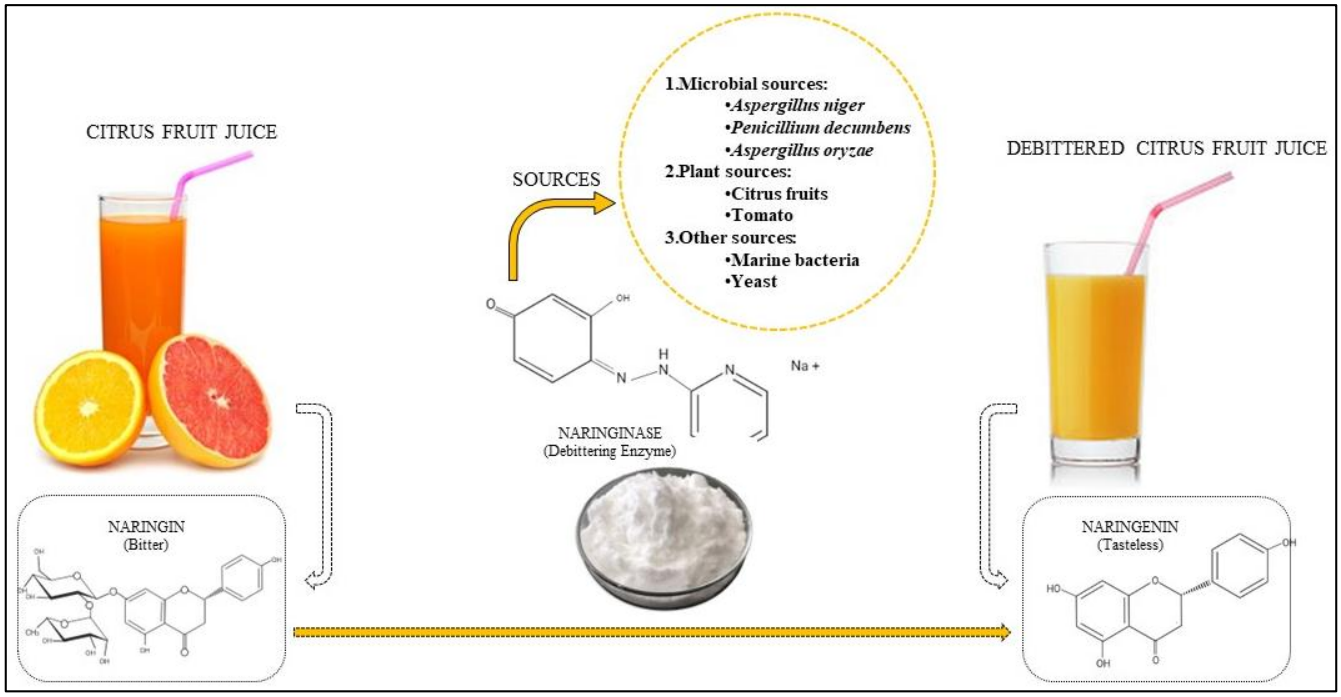

Fig 1: Debittering of citrus fruit juice

5. Conclusion

In conclusion, the immobilisation and isolation of naringinase have demonstrated significant promise for the debittering of citrus fruit juice. A number of methods, including ammonium sulphate precipitation, ion exchange chromatography, and gel filtering, have been used to isolate naringinase from microbial sources. Naringinase immobilisation has been found to provide a number of benefits, including increased stability, reusability, and simplicity in separating from the reaction mixture. For naringinase, a number of immobilisation strategies have been investigated, including as covalent binding, trapping, adsorption, and the use of nanoparticles as support materials. Numerous studies on the debittering of citrus fruit juice using immobilised naringinase have yielded encouraging results. It has been demonstrated that variables including enzyme loading, pH, temperature, and substrate concentration have an impact on the debittering process; therefore, it is essential to optimise these variables in order to maximise debittering efficiency. It has been proven effective to use immobilised naringinase to debitter a variety of citrus fruit liquids, including grapefruit, orange, and lemon. Due to the enhanced stability and reusability of immobilised enzyme, it has also been demonstrated that its usage offers the potential for scaling up and commercialization. It needs more scaling-up and optimisation research before it can be commercialized.

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