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REVIEW ARTICLE

Naringinase enzyme: production, properties, and immobilization

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Abstract: Naringinase is a multi-enzyme complex that consists of α-L-rhamnosidase and β-D-glucosidase, which work synergistically to hydrolyse naringin, a bitter flavonoid glycoside found in C*itrus* fruits. The production of naringinase is mainly carried out by microbial fermentation using fungi and bacteria. The enzyme yield and activity can be enhanced by optimizing fermentation conditions, such as pH, temperature, and substrate concentration. Characterization of naringinase includes determining its molecular weight, kinetic properties (Km and Vmax), optimal pH, temperature stability, and substrate specificity. These parameters are crucial for its effective application in industrial processes. Immobilization of naringinase on carrier materials, such as alginate, chitosan, or silica gel, improves enzyme stability, reusability, and activity under diverse conditions, making it more viable for large-scale industrial applications. Naringinase has numerous industrial applications. It is widely used in the food and beverage industry to debitter *Citrus* juices, improving flavour profiles. In the pharmaceutical and nutraceutical sectors, naringinase facilitates biotransformation of flavonoids, such as converting naringin into more bioactive compounds with antioxidant, anti-inflammatory, and anticancer properties. Further, this enzyme is utilized in wastewater treatment to break down phenolic compounds. The objective of this review article is to provide a comprehensive overview of the current advancements in the production, characterization, and immobilization of naringinase, along with its diverse industrial applications. The review aims to highlight the optimization strategies that enhance enzyme yield and activity, as well as emerging trends in enzyme immobilization techniques, which contribute to the enzyme's stability and reusability for various industrial processes. The advancements in production, characterization, and immobilization techniques continue to expand the industrial utility of naringinase, enhancing its effectiveness across various sectors.

Keywords: *Application, Characterization, Immobilization, Naringinase*

Introduction

Enzymes act as biological catalysts, which are usually made up of proteins. Enzymes can be isolated from cells and employed to catalyze a variety of commercially important industrial processes. In 1878, the term 'enzyme' was introduced by Wilhelm Kühne, a German biologist, to describe yeast's ability to make alcohol from carbohydrates. Enzymes are required in very low concentrations to catalyze reactions and accelerate reaction efficiency without being consumed (Robinson, 2015). The "lock-and-key" and "induced-fit" hypotheses have been proposed more than a century ago to express the structural interactions between enzymes and substrates is still accepted today to explain the process of catalysis (Agarwal, 2006).

Enzymes have certain fundamental and crucial properties that make them strong candidates for industrial operations. These properties include increased specificity and efficiency, the ability to recover enzymes after the product has been formed, and the ability to synthesize enzymes by using microbial platforms. For centuries, microbial enzymes have been used in a variety of applications such as food, feed, detergent, textiles, washing, tanning, and industries of pharmaceuticals, cosmetics, and fine chemicals (Yadav *et al.*, 2018). In industry, microbially produced enzymes are more preferred

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than those derived from animal or vegetable sources due to low production costs, easy large-scale production in an industrial fermenter, enhanced genetic manipulation potential, and rapid and enhanced growth rate. Such enzymes have already been commercialized in significant numbers of various industrial processes (Yadav *et al.*, 2018).

Food enzymes are the most extensively utilized enzymes in the industry to produce various food products (Kirk *et al.*, 2002). They are commonly employed in baking, drinking and brewing, dairy, dietary supplements, fats and oils, and artificial sweetener synthesis.

Naringin and Naringenin

Naringin is a common flavone glycoside found in Citrus fruits that has a variety of biological and pharmacological properties and is a bitter substance made up of naringenin, an aglycone, and neohesperidose linked to the hydroxyl group at C-7. It tastes bitter due to its glucose moiety. Naringin is currently on the list of flavouring compounds, which means it can be used in food without restriction. The efficacy of naringin in treating diabetes and its consequences has recently received more attention (Elias and Câmara, 2013) . Naringenin is a flavonoid that naturally occurs in a variety of edible fruits, including Citrus and tomatoes. It is soluble in organic solvents such as alcohol but insoluble in water. Naringenin has a wide range of biological effects on human health, including lowering the levels of lipid peroxidation biomarker molecules and protein carbonylation, increasing antioxidant defences, scavenging reactive oxygen species, modulating the activity of the immune system, and having anti-atherogenic and anti-inflammatory properties (Chen *et al.*, 2016, Salehi *et al.*, 2019). According to a recent comprehensive review, naringin has strong anti-inflammatory, anti-apoptotic, anti-ulcer, anti-osteoporotic, anti-carcinogenic, and antioxidant properties (Stabrauskiene *et al.*, 2022). Due to the steric hindrance of the scavenging group, naringin is less powerful than naringenin. Naringenin has several biological effects, including enzyme inhibitors, antioxidants, and anticancer properties, as well as being an anti-inflammatory drug (Stabrauskiene *et al.*, 2022).

Naringinase enzyme

Naringinase ($C_{11}H_9N_3O_2.Na^+$) is a member of an enzyme complex. It is a rhamnopyranosidase with the activity of L-rhamnosidase and D-glucosidase. Naringinase converts naringin to rhamnose and prunin (Figure 1), which accounts for one-third of the bitterness, then prunin is hydrolysed to tasteless naringenin and glucose (Puri, 2012). It is employed in the production of glycopeptide antibiotics, deglycosylation of flavonoids, removing the hesperidin haze from orange products, removing the bitterness from Citrus juice, and enhancing the aroma of grape juices. Although it can hydrolyze a variety of glycosides, including naringin, hesperidin, rutin, and 6-O-l-rhamnopyranosyl-d-glucopyranosides, it expressly converts naringin to naringenin. Naringinase occurs widely in nature and is produced externally by numerous microorganisms such as bacteria, fungi, yeast, and plant sources as well as mammalian tissues (Table 1). *Aspergillus niger, Aspergillus flavus, Staphylococcus xylosus, Williopsis californica* etc. are some organisms that can produce naringinase extensively. However, *Aspergillus niger* is regarded as the most significant source of naringinase for usage in food in terms of safety and practical growth techniques (Soares *et al.*, 2012).

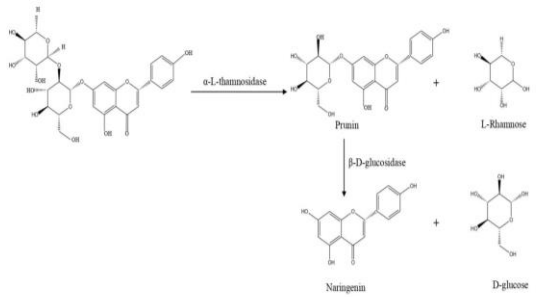

Figure 1. Schematic diagram of hydrolysis by the naringinase enzyme

Microbially produced naringinases are more suited as biocatalysts for numerous industrial applications due to low cost and ease of production, mainly (Soares *et al.*, 2012). Table 1 shows microorganisms that produce naringinase. Both non-enzymatic and chemical-based processing have some disadvantages in the debittering of food.

Significant temperatures and/or pressures are required to drive enzymatic reactions, resulting in high energy expenditures and large amounts of cooling water requirements in downstream processes. Higher chemical and energy usage, along with toxic by-products, harm the ecology during non-enzymatic and chemical debittering of food materials. Naringinase enzymes can essentially eliminate some or all these limitations in several situations

Table 1. Microorganisms that produce naringinase enzyme

Microorganism	Reference
Aspergillus aculeatus	Puri, (2012)
Aspergillus flavus	Radhakrishnan *et al.*, (2013)
Aspergillus foetidus	Ribeiro, (2011)
Aspergillus kawachii	Puri, (2012)
Aspergillus niger	Ribeiro, (2011) Srikantha *et al.*, (2017) Yadav *et al.*, (2018) Kanokpan *et al.*, (2008)
Aspergillus niger 1344	Puri and Kalra, (2005)
Aspergillus niger BCC 25166	Puri, (2012)
Aspergillus niger CECT 2088	Ribeiro, (2011)
Aspergillus oryzae	Zhu *et al.*, (2017)
Aspergillus oryzae JMU316 *Aspergillus oryzae* SS	Yadav *et al.*, (2018)
Aspergillus sojae	Ribeiro, (2011)
Aspergillus terreus	Puri, (2012)
Bacillus amyloliquefaciens 11568 *Candida tropicalis* *Clavispora lusitaniae*	Yadav *et al.*, (2018)
Clostridium stercorarium	Puri, (2012)
Cochiobolus miyabeanus	Ribeiro, (2011)
Lactobacillus acidophilus *Lactobacillus ulaiense*	Puri, (2012)
Penicillium caseicolum *Penicillium chrysogenum NRRL*	Yadav *et al.*, (2018)
Penicillium decumbens PTCC 5248	Norouzian *et al.*, (2000)
Penicillium glaucum *Penicillium roqueforti I* *Penicillium roqueforti II*	Yadav *et al.*, (2018)
Penicillium ulaiense	Puri, (2012)
Penucillium purpurogenum	Patil and Dhake, (2014) Yadav *et al.*, (2018)
Phanopsis citri	Ribeiro, (2011)
Pichia angusta *Pseudomonas paucimobilis* *Ralstonia pickettii*	Puri, (2012)
Rhamnus dahurica	Suzuki, (1962)
Rhizopus delemar	Yadav *et al.*, (2018)
Rhizopus nigricans	Ribeiro, (2011)
Serratia sp.	Yadav *et al.*, (2018)
Staphylococcus xylosus MAK2	Puri *et al.*, (2010b)
Willopsis californica	Chen *et al.*, (2013)

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Production of naringinases

A variety of media compositions have been investigated to develop effective media formulations that support the maximum synthesis of microbial naringinase. Kanokpan *et al.*, (2008) identified 348 fungal strains from 128 distinct host samples obtained from 11 different sources that can hydrolyze naringin at 28 °C and pH 5.8 on selected synthetic minimum medium containing 0.1% naringin. They found that, *Aspergillus niger* BCC 25116 was the most efficient naringinase producer. Optimization of its growth conditions using submerged fermentation resulted in maximum enzyme production (117.77 U/mg Protein), particularly when 0.1% naringin was present alongside $NaNO_3$ and rhamnose.

Another study showed that the response surface approach can be used to optimize the fermentation medium to increase the production of naringinase by *Staphylococcus xylosus* at the shaking flask level (Puri *et al.*, 2010a). The production of naringinase from *Staphylococcus xylosus* was highly influenced by many critical variables, including carbon (sucrose), nitrogen (sodium nitrate), pH, and naringin (inducer). When 10.0% sodium nitrate, 10.0% sucrose, pH 5.6, 1.58% biomass concentration, and 0.50% (w/v) naringin were used in the media, experimental naringinase production was 8.45 IU/ml (Yadav *et al.*, 2018).

The production of naringinase by a group of twelve filamentous fungi was also investigated by Mendoza-Cal *et al.*, (2010) utilizing agricultural wastes; grapefruit and orange rind were used as substrates since these materials contain naringin. *Aspergillus foetidus*, *Aspergillus niger*, and *Aspergillus niger HPD-2* each hydrolysed 81, 80, and 79% of the naringin from grapefruit peel respectively. When grown on grapefruit rind, *Aspergillus foetidus* displayed the highest volumetric activity (2.58 Uml-1) than that of the other species. In comparison to orange rind, grapefruit rind provided a significantly higher yield of naringinase by *Aspergillus foetidus* (Yadav *et al.*, 2018).

An extracellular synthesis of naringinase from a strain of the fungus *Rhizopus nigricans* was shown by (Shanmugam and Yadav, 1995). When rice and sugar were present in the culture medium, pH declined as the mycelia expanded. Significantly higher naringinase was produced after 50 hours of fermentation (Ribeiro, 2011).

Aspergillus niger MTCC 1344 produced extracellular naringinase in a complex medium composed of molasses, yeast extract, and salts (Puri and Kalra, 2005). Molasses and peptone were the most successful in achieving higher level of naringinase enzyme from Aspergillus niger MTCC 1344, compared to the other natural carbon and organic nitrogen sources investigated. *Aspergillus sojae* was identified by

Chang *et al.*, (2011) from a traditional Korean fermented soybean product as a higher naringinase producer. Naringinase enzyme produced by *Aspergillus sojae* was effectively converting the naringin to prunin with a 91% yield and a minuscule amount of naringenin (Ribeiro, 2011).

Characteristics of naringinase enzyme

Fungal naringinases have been biochemically characterized primarily using enzymes isolated from culture filtrates and commercial enzyme preparations from *Aspergillus* species, including *A. terreus*, *A. niger, and A. nidulans*. To assess naringinase activity, 348 fungal isolates were gathered from 11 different locations in Thailand and China. At 40 °C and pH 4.0, secondary screening was done by measuring the activities of L-rhamnosidase and D-glucosidase. The Czapek-Dox medium, which contains 0.1% naringin, was chosen as the ideal medium for the optimization of conditions for the synthesis of naringinase enzymes in submerged fermentation. The medium supplemented with 3.75 gL^{-1} rhamnose as a carbon source and 2.5 gL^{-1} sodium nitrate as a nitrogen source had the highest naringinase production (117.77 U mg^{-1}) (Puri, 2012). The molecular mass of the naringinase enzyme purified from the culture filtrate of A. niger 1344 by using gel filtration chromatography on a Sephadex G-200 column, was estimated to be around 168 kDa, and the molecular mass of the subunits was estimated to be 85 kDa by sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE). The optimum pH and temperature for the naringinase enzyme were 4.0 and 50 °C, respectively. The naringinase was stable at 37 °C for 72 hours, but after 96 hours of incubation at 40 °C, the enzyme showed 50% inactivation. At doses between 2.5 and 10 mM Hg2+, SDS, p-chloromercuribenzoate, Cu^{2+}, and Mn^{2+} totally inhibit the naringinase enzyme's activity, but Ca^{2+}, Co^{2+}, and Mg^{2+} showed very little inactivation even at high concentrations (10–100 mM) (Puri *et al.*, 2005).

There is evidence that naringin can cause *A. niger* MTCC 1344 to produce naringinase, using a step-by-step addition of modest amounts of naringin up to 7 days of fermentation in a complex medium containing molasses, peptone, and salts which gave the highest enzyme titer of 9.68 Uml-1 (Puri, 2012). When Ca2+ was introduced to the medium, an increase in the naringinase enzyme production rate was seen. In addition, rhamnose, hesperidin, naringin, and Citrus peel powder have all been reported as inducers of naringinase synthesis (Puri, 2012).

A Citrus post-harvest pathogenic fungus *Penicillium ulaiense*, which was first described in Taiwan, produced -L-rhamnosidase activity (Puri, 2012). They were able to synthesize L- rhamnosidase using *P. ulaiense* and rhamnose as the primary carbon source in a stirred-batch reactor for 12 days without pH regulation. Based on the above experimental results, good thermostability up to 60 °C and good operational stability in white wine were displayed by the purified enzyme (Rajal *et al.*, 2009). When naringinase was induced by various flavonoids and sugars including naringin, naringenin, rutin, quercetin, and hesperidin, the naringinase enzyme activity was found to be increased (Puri, 2012). When *P. decumbens* was cultivated at 30 °C with an initial pH of 7.0, utilizing 8.0 gl-1 rutin and 9.0 gL^{-1} di-ammonium hydrogen phosphate as carbon and nitrogen sources, respectively, the maximum enzyme activity was seen (Puri, 2012).

A culture of *Aspergillus sojae* that was obtained from fermented Korean soybeans recently showed naringinase activity (Chang *et al.*, 2011). When making soy sauce, this fungus has been utilized safely as a koji mould. The culture was raised in naringin-containing medim (0.5 gL^{-1}). The molecular weight of the isolated naringinase enzyme was 70 kDa. This naringinase enzyme's L-rhamnosidase activity was stable in the pH range of 5.5-8.0 and was best at a pH of 6.0, and naringin was successfully converted to prunin with a 91% yield and very little naringenin through enzymatic bioconversion. Using ultra-filtration and cation exchange chromatography on carboxy methyl (CM) cellulose, a naringinase enzyme was isolated from the culture filtrate of the fungal strain *A. flavus* MTCC 9606 (Chang *et al.*, 2011). SDS-PAGE results showed that the isolated naringinase enzyme had a molecular mass of 41 kDa. The naringinase enzyme's optimal pH and temperature were 11 and 50 °C, respectively. Alcohols and ethyl acetate inhibited the purified naringinase enzyme, indicating that it would not be a promising choice for enhancing the scent of grape wine (Puri, 2012).

The molecular mass of the naringinase enzyme purified from the culture A. aculeatus JMUdb058 was estimated to be around 348 kDa, and it was composed of four subunits with molecular masses of 100, 95, 84, and 69 kDa (Chen *et al.*, 2013). The smallest subunit was a α-L-rhamnosidase, while the three bigger subunits were β-D-glucosidases, according to mass spectrometric analyses. At about pH 4 and 50 °C, the naringinase and its α-L-rhamnosidase and β-D-glucosidase subunits all functioned at their peak levels; they were stable between pH 3 and 6 and below 50 °C. Naringin, aesculin, and a few other glycosides

may all be hydrolysed by this naringinase. The hydrolysis of naringin, aesculin, and a few other glycosides was accomplished by this naringinase. The total naringinase enzyme complex had a kcat/Km ratio of 14 034 s-1 mM-1 and a Km value of 0.11 mM. It possessed kcat/Km ratios of 14 146 and 7733 s-1 mM-1 for its α-L-rhamnosidase and β-D-glucosidase subunits, respectively, and Km values of 0.23 and 0.53 mM respectively (Chen et al., 2013).

The origin and fermentation conditions of naringinase affected its molecular weight. The naringinase from A. aculeatus had a molecular mass of 348 kDa and four subunits with a molecular mass of 100, 95, 84, and 69 kDa, three of which corresponded to -D-glucosidase subunits and one corresponded to a L-mannosidase subunit. In the case of A. niger, naringinase from A. niger van Tieghem MTCC 2425 had molecular weight bands of 10–20, 65–80, and 80 kDa while that from A. niger 1344 corresponded to a heterodimer of 168 kDa. Only one molecular weight band of 23 kDa was found for both L-rhamnosidase and β-D-glucosidase (Chen *et al.*, 2013).

Few naringinases of bacterial origin have been described, in part because the industry has taken a somewhat covert approach to the study due to their wide range of lucrative applications. The purification of L-rhamnosidases from *Bacillus sp*. *Clostridium stercorarium*, *Lactobacillus acidophilus*, *Sphingomonas paucimobilis*, and *Thermomicrobium roseum* have been described in recently published reports (Hashimoto *et al.*, 2003, Cardona *et al.*, 2006, Beekwilder *et al.*, 2009). The isolated L-rhamnosidase from *Bacillus sp*. R1 was a monomer with a molecular mass of 110 kDa and was most active at pH 8 and 50 °C. Divalent metal ions were necessary for the naringinase enzyme's activity (Puri, 2012).

In an another study, L-rhamnosidase was isolated and purified from Pseudomonas paucemobilis FP200 bacterial strain (Puri, 2012). They found that Ca^{2+} sped up the isolated naringinase enzyme's activity. Temperature and pH were ideal at 7.8 and 45 °C, respectively. The enzyme was purified using ammonium sulfate precipitation, dialysis, and affinity chromatography. An SDS-PAGE and isoelectric focusing tests were used to determine the enzyme's purity. When kept at 20 °C, the L- rhamnosidase isolated from this strain stayed stable for several months. In terms of Km, Vmax, and Kcat, p-nitrophenyl-L-rhamnopyranoside (pNPR) has values of 1.18 mM, 92.4 µmol/min, and 117,000/min, respectively (Puri, 2012).

Immobilization of naringinase enzyme

The enzyme naringinase is employed in a variety of industries to hydrolyze naringin to remove bitterness. There are several ways to lower the naringin level, including adsorptive debittering and treatment with -cyclodextrin. However, the enzymatic hydrolysis of naringin could be utilized as an alternate method due to several drawbacks of these debittering technologies. Treatment with naringinase acts as a promising technique because it improves organoleptic properties while preserving health-promoting properties. As a result, the use of naringinase enzymes in various industries is constantly growing. However, using free naringinase has several practical challenges, such as making it impossible to reuse the enzyme, making it sensitive to environmental changes, and separating the enzyme from the solutions. Enzymes are typically expensive due to the high isolation and purification costs than the cost of regular catalyst production. Since enzymes are made up of proteins, they are extremely vulnerable to different denaturing circumstances when separated from their normal surroundings. Because of their sensitivity to process variables like temperature, pH, and chemicals in trace quantities act as inhibitors. This sensitivity can lead to higher production costs. However, unlike traditional heterogeneous chemical catalysts, most enzymes work in homogeneous catalysis systems while dispersed in water, which results in product contamination and prevents their recovery for subsequent usage in the active state from most of the reaction mixtures. Therefore, using an immobilization strategy is one of the most effective strategies suggested to get over these restrictions (Kress *et al.*, 2002).

Numerous immobilization techniques are available for the immobilizatio of enzymes (Bodakowska-Boczniewicz and Garncarek, 2019). Immobilization is a technological process in which enzymes are fixed to or inside the solid support, resulting in a heterogeneous immobilized enzyme system (Figure 2). It is a well-known experimental technology to produce more stable, active, and recyclable enzymes. It is also a potent tool for designing environmentally friendly and sustainable production processes
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Nowadays, immobilized naringinase enzymes have proven to be highly effective for commercial use in many circumstances (Yu *et al*., 2021). Compared to the free enzymes in solution, they have several benefits, such as lower cost, greater stability, and the potential to be simply removed from the reaction mixture, allowing for the isolation of the pure product. As a result, an immobilized enzyme is bound to an inert, organic, inorganic, or insoluble substance, like

calcium alginate or silica. Furthermore, an enzyme's tolerance to various environmental changes, like pH or temperature, can be increased by bonding to a solid support (Homaei *et al*., 2013).

It is necessary to adopt inexpensive immobilization techniques to use immobilized naringinase enzymes in the food sector. The solid supports must also have reactive groups and be completely non-poisonous. The application of naringinase determines which immobilization technique is used. The hydrolysis of naringin utilizing immobilized naringinase has been used in a variety of ways to achieve debittering of C*itrus* fruit juice. Naringinase has been successfully immobilized on a variety of substrates, including polyvinyl alcohol, woodchips coated with glutaraldehyde, alginate beads, chitin, celite, and porous glass beads (Yu *et al.*, 2021).

Immobilization support material binding may be chemical or physical and involve covalent or weak bonds. Physical bonding between the enzyme and immobilization support material is often quite feeble and can hardly hold the enzyme to the carrier under industrial circumstances. The support can be made of synthetic resin, a biopolymer, or an inorganic polymer like zeolite or silica. Entrapment includes putting an enzyme inside a membrane device, such as a hollow fiber or a microcapsule, or a polymer network (gel lattice), such as an organic polymer or a silica sol-gel. The creation of the polymeric network is necessary for entrapment and must occur in the presence of the enzyme itself. Chemical bonding creates covalent

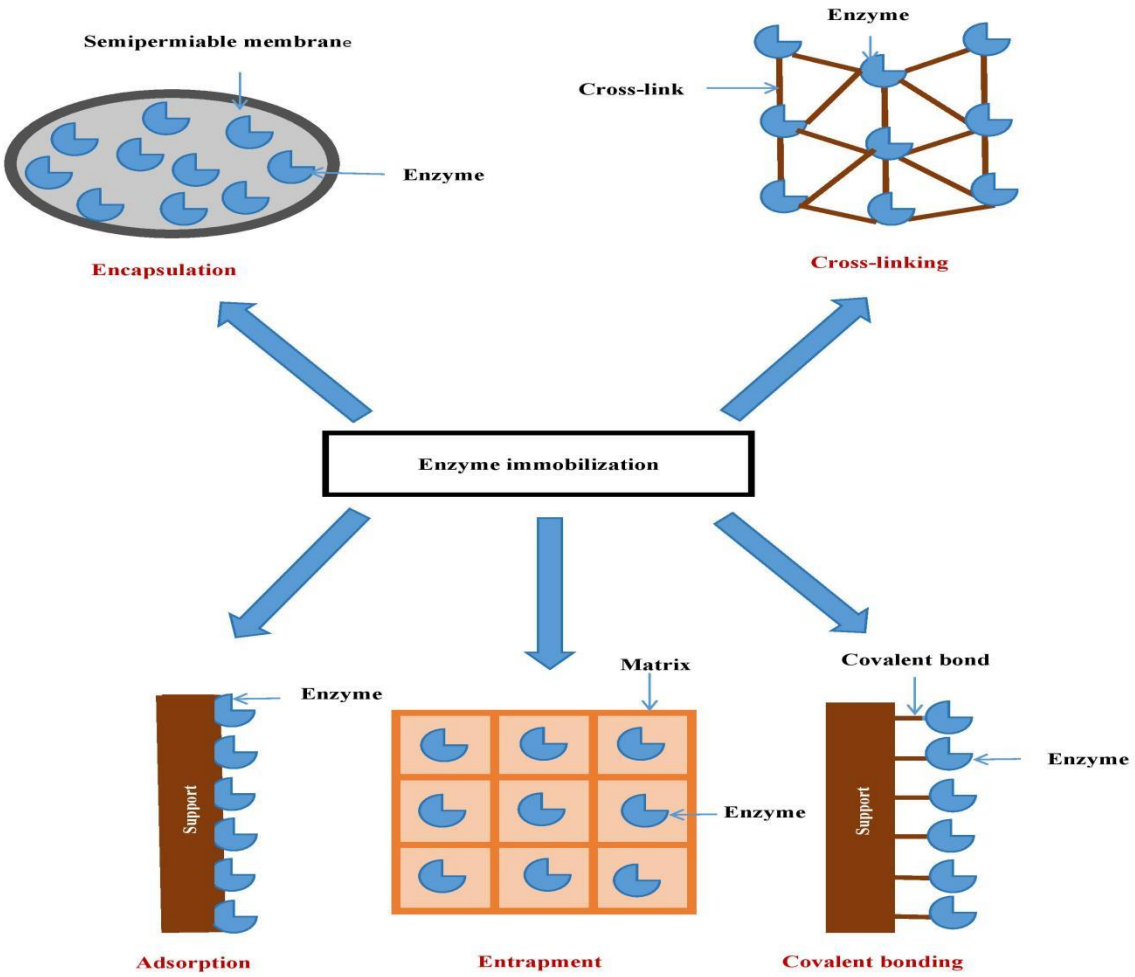

Figure 2. Diagram illustrating major immobilization methods.

bonds, which are necessary for a more stable attachment between the enzyme and support material and are typically created by reactions with the functional group on the surface of the protein (Bodakowska-Boczniewicz and Garncarek, 2019).

Research showed successful immobilization of naringinase from *Aspergillus aculeatus* on magnetic Fe_3O_4 nanoparticles (Bodakowska-Boczniewicz and Garncarek, 2019). Additionally, a technique for producing naringinase enzyme aggregates that are nano-magnetically cross-linked has been established. *Aspergillus terreus* naringinase subunit -l-rhamnosidase was covalently immobilized on three magnetic supports, including chitosan, polysiloxane/polyvinyl alcohol (POS/PVA), and dacron-hydrazide (Yadav *et al.*, 2024).

Chitosan is an effective support for immobilizing enzymes in the food sector. Because it is a non-toxic, biodegradable to harmless product, usable in various forms, including powders, flakes, and gels, and most importantly, has a high protein affinity (Bodakowska-Boczniewicz and Garncarek, 2019). When the carbonyl groups of glutaraldehyde and the amine groups of chitosan react, the structure of the chitosan support can be changed. Copolymer materials with better adsorption characteristics are made possible in this way. There is no solitary, ideal technique for immobilizing enzymes because of the diversity of biocatalysts and the wide range of uses for them following immobilization (Yadav *et al.*, 2024) .

The immobilization of enzymes on carriers with magnetic characteristics has drawn the attention of researchers recently because the enzyme can be quickly and easily separated from the reaction medium using a magnetic field, which is their main benefit. This is crucial when insoluble materials are present, as they are in grapefruit juice during the process. Covalent binding and physical adsorption are frequent techniques for immobilizing enzymes on magnetic carriers. Iron oxide (Fe_3O_4) is the most widely used substance for obtaining magnetic carriers (Homaei *et al.*, 2013).

Enzymes are frequently immobilized using natural carriers such as porous glass, silica gel, and different types of polysaccharides. Polysaccharide carriers make an excellent matrix for the immobilization of enzymes due to their abundance of chemically active groups, relatively large specific surface area, and significant adsorption capacity. Chitin, chitosan, and alginate were some of the natural carriers employed to immobilize naringinase. To improve the efficacy of the enzyme binding to the matrix, carriers employed for enzyme immobilization are frequently modified. Ionic polymers with NH_2 groups, such as polyethyleneimine (PEI), come into contact with the carrier that has aldehyde functional groups (PEI). Alternately, an aldehyde-containing reagent, such as glutaraldehyde (AG), is used to activate a polymer skeleton that contains amine groups, such as glutaraldehyde. Glutaraldehyde is the most often employed substance for altering the carrier's surface, regardless of the immobilization technique. The extra cross-linking of the catalytic protein with a carrier is frequently used in the case of immobilizing enzymes via adsorption to boost the stability of the resulting system. Glutaraldehyde is the substance that is utilized for boost stability most frequently. Dextran aldehyde is a different substance that also binds enzymes with a carrier (Homaei *et al.*, 2013).

The activation energy of naringinase was reduced roughly from 32.5 $kJmol^{-1}$ to 28 $kJmol^{-1}$ as a result of its immobilization on the magnetic polysaccharide carrier (Bodakowska-Boczniewicz and Garncarek, 2019). Stabilization with dextran aldehyde does not significantly change the activation energy compared to adsorption onto a solid support. The enzyme's catalytic capacity was raised as a result of the immobilization of naringinase from *Aspergillus niger* KMS by adsorption on the magnetic polysaccharide carrier (Bodakowska-Boczniewicz and Garncarek, 2019). The value of the naringinase preparation's activation energy was not considerably changed by additional dextran aldehyde coating (Bodakowska-Boczniewicz and Garncarek, 2019, Shilpa *et al.*, 2023).

The temperature affects the half-life of free naringinase cross-linked with dextran aldehyde depending on temperature. The value of the activation energy of the thermal deactivation process increased almost three times in comparison to the free enzyme as a result of the immobilization and subsequent cross-linking with dextran aldehyde of the naringinase preparation from *A. niger* KMS. The above findings suggest that the immobilized enzyme is more stable than the free naringinase. At 50 °C, the half-life of naringinase from *Aspergillus aculeatus* aggregates with lysine amine groups bound to Fe_3O_4 was approximately 7 hours (Bodakowska-Boczniewicz and Garncarek, 2019).

Naringinase has also been shown to immobilized with polyethyleneimine (PEI)-modified Fe_3O_4 magnetic nanoparticles using a co-precipitation method (Yu *et al.*, 2021). The immobilized naringinase exhibited an activity of 690.74 U per g of support under optimal conditions: pH 5.5, temperature of 35 °C, immobilization time of 4 hours, and an initial

naringinase activity of 406.25 Uml^{-1}. Further studies on the enzymatic properties revealed that the immobilized enzyme's activity peaked at 710.97 U per g of support when the reaction temperature was increased to 60 °C while maintaining the pH at 5.5. This activity was considerably higher than those reported in previous studies. Additionally, the naringinase loading efficiency and activity recovery of Fe_3O_4-PEI immobilized naringinase were 92.93% and 20.89%, respectively. Compared to the free enzyme, the immobilized naringinase demonstrated significantly enhanced pH and thermal stability. After ten cycles of reuse, the immobilized enzyme retained 60.58% of its relative activity. Moreover, after one month of storage at 4 °C, it preserved 87.52% of its relative activity. These results indicated that Fe_3O_4-PEI immobilized naringinase exhibited excellent operational and storage stability, highlighting the potential of Fe_3O_4-PEI magnetic nanoparticles as a promising material for enzyme immobilization, offering enhanced enzyme reusability and stability for future applications in the immobilized enzyme field.

Puri, (2012) used naringinase-containing calcium alginate beads to treat grapefruit juice, and after 180 minutes at 55 °C and 220 rpm, 83.84% of the naringin was hydrolysed. The soluble enzyme degraded 65.53% of naringin in comparable circumstances. The immobilized and soluble naringinase enzymes were found to have Michaelis-Menten kinetic constant values of 9.75 and 20 mM, respectively. An immobilization yield of 90% was achieved after naringin hydrolysis using naringinase immobilized in calcium alginate (2% and 3%) beads. In another study, naringinase from *Penicillium* sp. was covalently bound to wood chips and immobilized (Puri and Kalra, 2005). The immobilization method was optimized on glutaraldehyde-coated woodchips (600 mg woodchips, 10 U naringinase, 45 °C, pH 4.0, and 12 hours), adding 1% of glutaraldehyde cross-linking. Naringinase was adsorptively immobilized on Celite at ideal conditions, and naringinase adsorbed on celite exhibited a residual activity of 83% (Yadav *et al.*, 2024). Polyvinyl alcohol (PVA) is a synthetic polymer that is inexpensive and safe from microbial contamination. PVA-alginate beads were created at high temperatures (>80 °C) for thermal, mechanical, and chemical stability (Yadav *et al.*, 2024). Alginate and sodium sulfate treatment of the beads not only prevented agglomeration but also created beads with a high gel strength and provided enzyme protection from boric acid inactivation. *Penicillium decumbens* naringinase was immobilized in PVA (10%) alginate beads of three different sizes (1-3 mm), each with a different alginate concentration (0.2-1.0%). The swelling ratio within the beads, the enzyme's activity, and the immobilization yield during naringin hydrolysis were all examined. The optimal pH and temperature for the PVA-alginate-immobilized naringinase were 4.0 and 70 °C. At pH 4.0 and 70 °C, the greatest naringinase activity yield in 2 mm PVA (10%)-alginate (1%) beads was 80%. With a retention activity of 70% over eight successive batch runs, the immobilized naringinase's residual activity was monitored. Ninety percent of the initial activity was still present in the immobilized biocatalysts after 6 weeks of storage in an acetate buffer at pH 4 and 4 °C (Puri, 2012, Shilpa *et al.*, 2023).

Sodium alginate (2% w/v) was identified as the most effective medium for immobilizing naringinase. After immobilization, 30 U of naringinase was able to achieve 82% hydrolysis of naringin within 3 hours (Puri *et al.*, 2005). This optimized alginate matrix resulted in a 60% reduction in bitterness. The enzyme's functionality across a wide pH range offers flexibility in debittering kinnow juice, while its thermostability could help lower the costs of the process. The study also found that alginate promotes faster equilibrium without disrupting the movement of naringin and its hydrolysis product, naringenin prunin, while preserving mechanical stability, indicating its potential for commercial application (Kumar *et al.*, 2021, Yadav *et al.*, 2024).

Luo *et al.*, (2019) conducted a study on the immobilization of naringinase using porous silicon materials, with silica material with a pore diameter 7.7 nm (SBA-15) and found to be particularly effective following silanization and modification. The optimal conditions for immobilizing naringinase included an initial enzyme activity of 89.04 UmL^{-1}, a temperature of 40 °C, a pH of 3.5, and a refrigerated storage time of 4 hours. Under these conditions, the highest naringinase activity recorded was 517.43 Ug^{-1}. The immobilized naringinase demonstrated excellent stability, retaining 61.81% of its enzymatic activity even after eight reuse cycles. Moreover, after 30 days of storage at 4 °C, the enzyme retained 80.95% of its activity. Thus, SBA-15 shows promise as a novel material for enzyme immobilization, potentially benefiting naringinase applications and the fruit juice industry.

The potential of membrane technologies in the food and beverage sector is generally recognized nowadays. Since membranes function as selective barriers that make it easier to separate the biocatalysts from the reaction product and have a large surface for enzyme loading, they are also intriguing supports for the immobilization of enzymes. Enzymatic membrane reactors (EMR) are, moreover, particularly helpful in

reactions when product inhibition is a possibility. Immobilization using membranes is a fascinating possibility because rhamnose reduces naringinase activity. EMRs act as selective barriers that can separate the enzyme from the reaction products, including rhamnose. Therefore, by removing rhamnose from the reaction system, the membrane helps to reduce product inhibition and maintains the enzymatic activity of naringinase.

Entrapment, adsorption, cross-linking, and covalent attachment are just a few of the known instances of enzyme immobilization techniques in or on membranes. The use of membrane technology to immobilize naringinase is not widely documented. The fouling-induced method, one of the simplest membrane immobilization techniques, relies on the intentional encouragement of fouling using pressure-driven filtration to capture or adsorb enzymes in the membrane. Fouling is affected by a variety of factors, including feed conditions (such as concentration and pH), process variables (such as applied pressure), and membrane characteristics (such as pore size and configuration) (González-Temiño *et al.*, 2021).

The process of immobilization brought on by fouling involves physical interactions between the membrane and the enzyme, principally Van der Waals forces, hydrogen bonds, and electrostatic forces. Due to the tenuous connections made with the carrier, the biocatalysts can be easily eliminated by washing it or by reaction cycles. Enzyme molecules adsorbed on the membrane can be further cross-linked using a functional reagent like glutaraldehyde to reduce the possibility of desorption. Using the fouling-induced method and glutaraldehyde cross linking, *Penicillium decumbens* naringinase has been successfully immobilized on polyethersulfone ultra-filtration membranes. With a membrane pore size of 10 kDa, in reverse mode configuration, a transmembrane pressure of 0.2 MPa, 0.3 gL^{-1} of the enzyme at pH 5, and cross-linking with 0.25% glutaraldehyde, the best results for naringin conversion (73%) were obtained. The grapefruit juice's pH, soluble solids content, or titratable acidity were all maintained over at least three cycles of debittering it with the EMR utilizing immobilized naringinase. Only a minor portion of the juice's antioxidant capacity was impacted. The juice's naringin content was cut in half (González-Temiño *et al.*, 2021).

Application of Naringinase

Debittering of Citrus Juices

Some naturally occurring food materials are undesirable for several reasons, including their undesirable flavour and anti-nutritional qualities, such as naringin and limonin. This has caused significant issues with bitterness and delayed bitterness onset in *Citrus* fruit juice processing. The bitterness affects consumer acceptability, which has been a long-standing problem (Yadav *et al.*, 2018). When *Citrus* fruit juices are packaged, there has been research on the idea of utilizing enzymes to remove these substances in situ. A naringinase from a fungus was immobilized by (Soares and Hotchkiss, 1998) on cellulose acetate film to create an active packaging film that converted the bitter compound naringin into the non-bitter compound naringenin.

The bitter taste of naringin has been reported to be apparent in water solutions at concentrations of more than 20 $mgml^{-1}$, while grapefruit juices only exhibit bitterness at concentrations greater than 300–400 $mgmL^{-1}$. The lowering of naringin concentration by naringinase hydrolysis is one potential technology with industrial use for retaining the health characteristics and enhancing the economic value of citrus juices, namely grapefruit juice. With naringinase confined in alginate beads, grapefruit juice's bitterness was lessened, resulting in 84% naringin hydrolysis. Naringinase immobilized in k-carrageenan is able to reduce naringin by 95% (Ribeiro and Ribeiro, 2008). Debittering kinnow juice with naringinase enclosed in calcium alginate beads, achieved a 60% decrease in the naringin level (Puri *et al.*, 1996). Pedro *et al.*, (2007) and Ribeiro *et al.*, (2010) carried out a new method employing naringinase encapsulated in calcium alginate beads to lessen the bitter flavour of juices. Naringin enzymatic hydrolysis in *Citrus* juices was controlled using response surface methods. By using the response surface method, the highest naringin conversion of 81% was achieved with 205 MPa and 60 °C throughout a 30-minute operation (Ribeiro, 2011).

Enhancement of aroma in wine

Pectinases and xylanases are frequently employed to enhance the procedure and increase clarifying since wine is produced by the maceration and juice extraction of grapes. In addition to these, naringinases are utilized to improve the aroma because they hydrolyze the aglycone moiety of grape secondary metabolites and subsequently release the aglycone moiety; urease is utilized to prevent the formation of ethyl carbonate, a possible mild carcinogen, which results from the spontaneous reaction between urea and ethanol; and glucose oxidase is utilized to lessen the alcohol content by reducing the amount of glucose available for fermentation (Yadav *et al.*, 2018).

Antibiotics production

Enzymatically, chloropolysporins A, B, and C could be converted to deglycosylated derivatives. Chloropolysporin C and β-lactam antibiotics worked together synergistically to combat methicillin-resistant *Staphylococcus* strains. Chloropolysporins have potent antibacterial effects on Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, and they also inhibit Gram-positive enterobacteria that grow in anaerobic environments. With the help of naringinase's rhamnosidase activity, the new glycopeptide antibiotic chloropolysporin from the *Faenia interjecta* was successfully deglycosylated (Ribeiro, 2011). They derhamnosylated Chloropolysporin B to produce the glycopeptide antibiotic chloropolysporin C.

Steroids Biotransformation

Naringinase uses enzymatic hydrolysis of the fenugreek seeds (*Trigonella foenum graecum*) to make sapogenins and diosgenin, which are precursors of clinically effective steroid medicines. The immobilized pectinase and naringinase could be employed twice in this method without reducing the yield of diosgenin (Elujoba and Hardman, 1987). Numerous medications and drug precursors can be made by using the L-rhamnosidase produced by the naringinase enzyme. Diosgene (a saponin) is hydrolysed by L- rhamnosidase to create L- rhamnose and diosgenin, which are employed in the manufacture of therapeutically effective steroid medications like progesterone (Ribeiro, 2011).

Rhamnose production

Naringinase's L-rhamnosidase activity hydrolyses substrates such as naringin to generate L-rhamnose. Rhamnose functions as a chiral intermediary in the organic synthesis of essential drugs and plant defence mechanisms. The terminal L-rhamnose in the glycosides is released by the enzyme L-rhamnosidase (Ribeiro, 2011). Therefore, the production of L-rhamnose could benefit from L-rhamnosidase. To create rhamnose, Danials *et al.*, (1990) used a partially purified preparation of naringinase that had a high rhamnosidase activity and a low glucosidase activity.

Ginsenosides production

Ginsenosides are the major active pharmacological components of ginseng and are also known as steroid-like saponins (Kim *et al.*, 2017). Ginsenosides are heavily glycosylated in nature. By cleaving glycosyl groups, glycosidases like naringinase can create ginsenosides with enhanced activity. Side effects could result from chemical synthesis, moderate acid hydrolysis, or alkaline cleavage used to create small saponins and saponin metabolites (Ribeiro, 2011).

Conclusion

Naringinase is a natural multi-enzyme system consisting of α-L-rhamnosidase and β-D-glucosidase activities. It is capable of hydrolysing naringin, a bitter glycoside commonly found in *Citrus* fruits. Naringinase is primarily produced through microbial fermentation, employing fungi and bacteria under optimized conditions—such as specific pH, temperatures, and substrate concentrations—to maximize yield and enzymatic activity. Understanding enzyme properties such as molecular weight, kinetic parameters (K_m and V_{max}), optimal pH and temperature stability, and substrate specificity are crucial for its effective utilization in various industrial processes. Immobilization of the enzyme on support materials such as alginate, chitosan, or silica gel has showed marked improved in its stability, reusability, and functional activity under diverse conditions, thus enhancing its suitability for large-scale applications. The enzyme's industrial applications are extensive and diverse: it is employed in the food and beverage industry to mitigate the bitterness of *Citrus* juices and refine flavor profiles. In the pharmaceutical and nutraceutical sectors, naringinase facilitates the biotransformation of flavonoids, producing more bioactive compounds with significant antioxidant, anti-inflammatory, and anticancer properties. Additionally, naringinase is important in environmental management, where it contributes to the degradation of phenolic compounds in wastewater. In summary, ongoing advancements in the production, characterization, and immobilization of naringinase tend to broaden its industrial applications and enhance the efficiency of the enzymatic activity. These advancements highlight naringinase's importance in flavour enhancement, health benefits, and environmental sustainability, affirming its role as a versatile and indispensable enzyme across multiple industries.

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