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REVIEW ARTICLE

Purification and Characterization of Microbial Naringinase for De-Bittering of Citrus Juices by Hydrolysis of Naringin: Current Status and Future Perspectives

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ABSTRACT
Naringin, a flavonoid, is naturally present in citrus fruits and therefore, extracted citrus juices. It is among the major contributors to the bitterness of citrus juices. Naringinase is an enzyme having ability to hydrolyze the naringin into a tasteless compound naringenin and therefore, may be used to reduce the bitterness of citrus juices in a controlled manner. As compared to chemical processing, naringinase based hydrolysis and treatment of juice provides several advantages. Naringinase may provide an alternate to the chemical processing of citrus juices. Moreover, the end product of naringin hydrolysis is naringenin, which have high therapeutic potential. Various microbes have been studied for production of naringinase and the enzyme naringinase has been evaluated for hydrolysis of naringin in citrus juices. In current article, various methods used for purification of naringinase and its characteristics have been discussed which are crucial in understanding the potential of microbial naringinase. This review article presents current status and future perspective of the naringinase purification, characterization and use of this industrial important enzyme for hydrolysis of naringin.
Keywords: *Naringin, Naringinase, Citrus juices, Debittering, Dood industry.*

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INTRODUCTION

Naringin is a flavonoid, which is known to contribute the bitterness to the extracted citrus juices. Citrus fruits naturally contain the naringin. The delayed commencement of bitterness in citrus fruit juice affects its consumer acceptability [1]. The enzyme, naringinase hydrolyses the flavonoid "naringin". The hydrolysis lead to production of flavanone "naringenin" which is non-bitter in taste. Naringinase have two subunits, (1) α-L-rhamnosidase and (2) β-D-glucosidase [2,3]. Naringinase mediated hydrolysis of naringin completes in two sequential steps involving action of "α-L-rhamnosidase" to produce prunin with rhamnose followed by second reaction in which purnin is further hydrolyzed by "β-D-glucosidase" to produce naringenin with simultaneous release of glucose [4-6]. Naringinase has been studied for its potential use in various industries [7,8]. The enzyme naringinase has found its major use in food industry particularly involved in processing of juices extracted from citrus fruits (Fig. 1), where the flavonoid naringin mediated bitterness requires to be removed [9-13]. Also, this enzyme has been found to strengthen the flavor of wines [14,15]. The naringenin, rhamnose, prunin and glucose produced during naringin hydrolysis have been found useful in different industries [9,16,17].

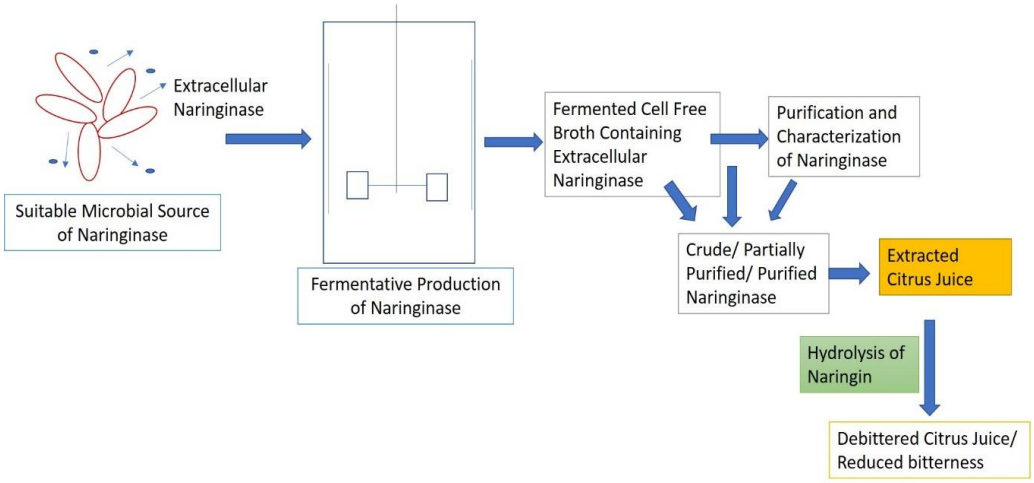

Figure 1: Schematic presentation showing general process of naringinase production and use for hydrolysis of naringin in extracted citrus juice for de-bittering.

Majority of research on naringinase focuses on fungal sources. Some promising bacterial sources of naringinase have also been reported. Characterization of crude, partially purified and purified naringinase has been reported. Crude naringinase has proved useful in debittering of citrus juices by hydrolysis of naringin (Fig. 1). This enzyme needs to be characterized more intensively as the progress of research in the area of naringinase seems to be slow according to its industrial potential. This review article focuses on characterization of crude, partially purified and purified naringinase. Characterization of naringinase is considered important before assessment of its potential of naringin hydrolysis. This article covers recent advances in the naringinase research and may provide important insight into production of microbial naringinase as well as characterization for citrus juice processing. This is first article of its type and may benefit the researchers working in the area.

PURIFICATION OF NARINGINASE

Purification of enzyme is considered an important step in fermentation technology to assess the characteristics of enzymes and their applications. Naringinase is a very specific enzyme and researchers have studied the properties of enzyme in crude form, partially purified form and purified form as well. Kumar et al. [18] have reported production and purification of naringinase from *Micrococcus* sp. Authors performed ultrafiltration using membrane having 10 kDa molecular weight cut-off followed by ammonium sulphate precipitation and SDS-PAGE in a sequential manner. The ammonium sulphate precipitation was performed in range of 20-80% (w/v) saturation. Sodium citrate buffer (1M; pH 4.0) was used for dissolving the precipitates and also dialysis.

Purification of naringinase from *Bacillus amyloliquefaciens* has been discussed [19], Th purification involved use of size exclusion chromatography (Sephadex G-15) column. The elution buffer used was containing phosphate buffer (20 mM; 0.1 M NaCl; pH 7). Further, authors used anion exchange chromatography in a successive manner to significantly improve the purification level of enzyme. The DEAE-sepharose based column (anion exchange) was used. Citrate buffer (20 mM; pH 5.5; NaCl 1M) was used for elution of naringinase [19]. Recently, Borkar et al. [20] partially purified the seven days fermented broth. The harvested broth was passed through ultrafiltration membrane (30 KDa, molecular weight cut off) followed by precipitation using ammonium sulfate (20-80% saturation). The filtrate containing enzyme was mixed with appropriate ammonium sulphate solution. The suspension was kept for 30 hours at 4°C followed by centrifugation (30 minutes; 4000 rpm). The precipitated proteins were dissolved in 0.1 M sodium acetate buffer (pH 4.0) followed dialysis against 10 mM sodium acetate buffer having pH 4.0. With a total protein content of 492.8 mg, more activity (86%) was recovered in the ammonium sulfate solution at 80% saturation. Thereafter, the enzyme was purified using anion exchange chromatography. Among three screened resins, the UNOSPHERE Q was found most suitable for purification. Linear gradient elution based on phosphate buffer (0.05 M; pH 7.0) and NaCl (1M) was performed. After ion exchange chromatography, 9.92-fold purification was obtained with 17% activity recovery. Earlier, Puri and Kalra [21] described the purification of naringinase from *Aspergillus niger* 1344. The enzyme was purified by ultrafiltration (with a 30 kDa cut-off), ammonium sulfate precipitation followed by ion exchange as well as gel filtration chromatography. A 38-fold purification of

enzyme was obtained with recovery of 19%. The purified naringinase was studied for various characteristics [21]. Purification of naringinase enzyme (from *Aspergillus brasiliensis* MTCC 1344) using three phase partitioning technique has been reported earlier [22]. A purification fold of 4.2 was obtained by treating the crude extract at 28±2°C and ammonium sulfate (30%; w/v) with 1:1 ratio of t-butanol [22]. Production, purification and thereafter, characterization of enzyme naringinase from bacterial source (*Bacillus subtilis* strain BSnari) has been reported [23]. Authors partially purified the naringinase using ammonium sulfate followed by gel filtration. A purification fold of 8.95 was obtained along with specific activity of 215.106 U/mg [23]. Naringinase from *Candida tropicalis* has been purified and characterized by Saranya et al. [24]. The culture supernatant was processed through ultrafiltration using a membrane (50-kDa molecular weight cut-off). The resulting retentate was studied for precipitation of proteins by different concentrations of ammonium sulphate followed by dialysis of precipitated proteins against phosphate buffer (0.05 M, pH 7.0). Higher naringinase activity was obtained with 65% saturation of ammonium sulfate precipitation. The enzyme was further purified by using anion-exchange chromatography and gel filtration chromatography. DEAE-cellulose (anion exchange) and DEAE-Sephadex (gel filtration chromatography) was used for purification of naringinase [24].

Ni et al. [25] successfully purified naringinase from *Aspergillus niger* and conducted a characterization study to assess its effectiveness in reducing bitterness in citrus juice. The purification process involved sulfate fractionation followed by chromatography. The SDS-PAGE analysis showed a subunit mass of approximately 65.5 kDa, while gel filtration chromatography indicated a molecular weight of roughly 131 kDa for naringinase [25]. Chen et al. [26] reported purification of naringinase enzyme from fungal source *Aspergillus aculeatus* JMUdb058 and also characterized for different aspects. Naringinase from *Penicillium purpurogenum* was partially purified by Patil and Dhake [27]. The culture filtrate was centrifuged (5000 g, 20 min, 4ºC) and supernatant was used for ammonium sulfate precipitation (80% saturation). The precipitate obtained after ammonium sulfate treatment was dissolved appropriate amount of sodium acetate buffer (pH 5.5; 0.1M) and further, the residual ammonium sulphate from the suspension was removed by dialysis against same sodium acetate buffer for a period of 18 h. The partially purified enzyme was assessed for its application in debittering of citrus juice [27]. Production of naringinase as well as purification fungal source *Penicillium decumbens* PTCC 5248 was reported by Norouzian et al. [28]. Authors obtained 24-fold purification on fractionation with isopropanol. The partially purified naringinase was characterized for various properties.

Balaraman et al. [29], recently, reported production and purification of naringinase from *Bacillus amyloliquefaciens.* Authors used waste tofu water for production of enzyme and simultaneously performed purification (in-situ) with the help of natural deep eutectic solvents (NADES). The NADES mediated extracted enzyme was further processed through size exclusion chromatography and anion exchange chromatography with specific activity (naringinase) of 3516 U/mg. Awad et al. [30] reported production of *Aspergillus niger* naringinase and extraction of crude enzyme. The enzyme preparation was used for immobilization and hydrolysis of naringin. The enzyme naringinase has been purified in partial as well as complete for further characterization and application of purified naringinase. Though, the pace of research on purification and characterization of naringinase is not according to the industrial potential of this enzyme. More studies are required on purification and application of naringinase.

CHARACTERIZATION OF NARINGINASE

Naringinase produced from various microbes has been characterized in crude, partially purified form and higher (full/ complete) purified forms. Naringinase from *Bacillus amyloliquefaciens* 11568 was characterized by Zhu et al. [19]. The optimal conditions for naringinase activity have been determined, with a pH of 7.5 and a temperature of 45°C being the most effective. The study also revealed that the enzyme formulation maintains its stability below 45°C across a pH range of 3.5 to 8.5. Furthermore, the kinetic parameters for naringinase were characterized, yielding Km and Vmax values of 0.95 mmol/L and 3847.3 mmol/(L·min), separately. Naringinase has been also assessed for its capacity to hydrolyze naringin, it was found that a naringinase concentration of 4 U/ml is sufficient to completely break down naringin, significantly diminishing the juice's bitterness. Crude naringinase from *Aspergillus niger* has been characterized [31]. The optimal pH for both naringinase and α-rhamnosidase activities was found to be identical, at 5.0. Further, the optimum temperature for both catalytic activities was also found to be same at 60°C [31]. Naringinase from *Aspergillus brasiliensis* MTCC 1344 was purified using a three-phase partitioning technique [22]. A purification fold of 4.2 was attained by subjecting the crude extract to ammonium sulfate at a 30% (w/v) concentration, combined with same of the volume of t-butanol, & maintaining the process at a temperature of 28 ± 2°C [22]. The purified enzyme exhibited optimal activity at 60°C. The kinetic parameters, Km and Vmax , were reported to be 3.21 mM and 321 U/ml,

respectively, when naringin served as the substrate. Additionally, the purified naringinase showed inhibition by $Hg^{(2+)}$ and chelating agents (EDTA and SDS) at 10 mM concentration.

Naringinase from *Aspergillus oryzae* NYO-2 was used for production of prunin and naringenin [32]. With optimal conditions of enzyme, authors reported conversion of 19 mM naringin into 14.06 mM prunin & 1.97 mM naringenin [32]. Partially purified naringinase has been shown to have molecular weight of 73 (KDa) and the enzyme was capable of reducing the bitterness by around 33-36%, by hydrolysis of naringin after 4 h at 40-50°C. The purified naringinase from *Candida tropicalis* revealed two bands of 73 KDa (molecular weight) and 78 KDa (molecular weight) in SDS-PAGE [24]. The enzyme had optimal pH of 3.0 while it retained good activity at pH 5.0 also. The optimal temperature was found to be 25°C, Km value as 0.19 mM and Vmax was found to be 0.1 U/ml [24]. Sahota and Kaur [33] characterized naringinase from *Clavispora lusitaniae* for various properties. Km value of 1.0 mM and Vmax value of 28.56 was obtained for naringinase. The divalent cations Cu^{2+} and Ca^{2+} were found to inhibit the naringinase activity completely, while, Mn^{2+} and Zn^{2+} were found to stimulate the activity of enzyme [33]. Ribeiro [8] has reviewed characteristics of naringinase from various microbial sources. Various characteristics of crude naringinase from *Aspergillus flavus* has also been reported [34]. The enzyme revealed optimal activity at pH 4.5 while optimal temperature was found to be 50°C. among metal ions, Ca^{2+} and Mg^{2+} ions were found to support naringinase production whereas Fe^{2+} and Mn^{2+} ions were shown to have inhibitory effect on growth of *A. flavus* and also enzyme production. Similar results have been observed for partially purified naringinase of *Penicillium decumbens* PTCC 5248 [28]. The optimal pH was found to be 4.5 while temperature optimal for naringinase activity was recorded as 55°C. Authors reported a K_m value of naringinase for "naringin" as 1.7 mM [28]. Purified naringinase from *Aspergillus niger* has been shown to have "molecular weight" of 131 kDa, with subunit molecular weight of 65.5 as revealed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) [25]. The optimal pH range was found to be 4.5-5.0 and the optimal temperature was in the range of 45-55°C [25].

Naringinase from *Aspergillus niger* FFCC uv-11 was immobilized by silica materials and charecterized [35]. The immobilized enzyme was shown to have optimal temperature of 45°C and pH of 4.5. Authors reported the "binding efficiency, activity recovery rate and specific activity" of the immobilized naringinase as "63.66%, 87.64% and 517.43 U g^{-1}", respectively [35]. Naringinase from *Micrococcus* sp. has been purified and characterized [18]. The enzyme revealed optimum pH of 6.0, while 37°C was found to be optimal temperature. Further, the molecular weight was observed as 48 KDa.

Characterization of purified naringinase of *Aspergillus aculeatus* JMUdb058 has been reported earlier [26]. The enzyme was reported to have molecular weight of 348 KDa having four subunits with different individual molecular weight [26]. The naringinase and its subunits were shown to have optimum pH of 4 while optimal temperature was found to be 50°C. Although, the enzyme subunits were found to stable between pH 3-6 [26]. The K_m value was observed as 0.11 mM for complete enzyme complex [26]. Various properties of naringinase enzyme from *Aspergillus niger* CECT 2088 has been discussed by Busto et al. [9]. Authors immobilized the naringinase in a poly(vinyl alcohol) "(PVA)" hydrogel and the enzyme (immobilized) showed optimal temperature of 70°C. The activation energy of immobilized naringinase was decreased as compared to free enzyme (8.09 $kJmol^{-1}$) and reported to be 6.36 $kJmol^{-1}$ [9]. The partially purified naringinase enzyme from fungal strain, *Aspergillus niger* van Tieghem MTCC 2425 showed optimum pH of 5 at temperature of 50°C [20]. Further, the enzyme retained its activity (naringinase) completely up to 150 days (storage at 4°C). Authors reported that naringinase (in partially purified form) revealed molecular weight bands of various sizes in SDS-PAGE [20]. Bodakowska-Boczniewicz and Garncarek [36] reported immobilization of microbial naringinase from fungi *Penicillium decumbens* using chitosan microspheres. The free as well as immobilized enzyme showed optimum pH of 4.0. Balaraman et al. [29] recently, reported production and purification of naringinase from *Bacillus amyloliquefaciens.* Authors determined the K_m and V_{max} of naringinase to as $1.5x10^{-3}$ mmol min^{-1} and 2.76 µmol min^{-1}, respectively. Awad et al. [30] reported production of *Aspergillus niger* naringinase and extraction of crude enzyme. The enzyme preparation was used for immobilization and hydrolysis of naringin. The immobilization of enzyme increased its thermal stability from 50°C to 70°C. it was found that enzyme in immobilized state was more stable as compared to free enzyme. Further, the free enzyme revealed optimum pH of 4.5 while, the immobilized naringinase showed optimal pH of 4.0 [30]. Similar characteristics of immobilized (on magnetic Fe_3O_4 nanoparticles) naringinase from *Aspergillus aculeatus* has been discussed by Xiao et al. [37]. It was found that immobilization did not affect the ideal pH and temperature values of naringinase. Gupta et al. [38] reported characterization of naringinase from *Aspergillus niger.* The enzyme was also immobilized and assessed for debittering of citrus juice. The optimal pH of enzyme was found to be 4 along with temperature of 70°C. The "Michaelis constant" constant was reported to be 16.75 µg/ml. Moreover, the enzyme reduced naringin content by 79.76% in

120 minutes [38]. The enzyme purified from *Aspergillus niger* revealed subunit molecular weight of around 65.5 KDa [25]. The optimal pH was found in a range of 4.5 to 5.0, while optimal temperature was reported to be in range of 45 to 55°C. Igbonekwu et al. [39] also reported characterization of naringinase from *Aspergillus niger.* Optimal pH 0f 3.5 and temperature of 50°C was obtained. Further, the K_m and V_{max} was obtained to be as 5.8 mg/ml and 1111.11 µmol/ min, respectively.

Karuppaija et al. [40] reported characterization of naringinase from *Rhizophus stolonifera.* The crude naringinase showed higher activity at pH 4.0 and temperature of 65°C. Authors reported V_{max} of the crude enzyme (naringinase) as 3.125 µmol/mL while "Michaelis constant" as 3.076 mg/mL. Among metal ions, Mn^{2+}, Cu^{2+} and Ba^{2+} lead to increase in the naringinase activity, but, Mg^{2+}, Zn^{2+}, Hg^{2+}, Ca^{2+} and Na^{+} lead to decreased enzyme activity [40]. Puri and Kalra [21] reported characterization of purified naringinase from *Aspergillus niger* 1344. The purified enzyme showed optimal pH of 4.0 at temperature of 50°C. Further, the enzyme showed stability for 72 h at 37°C while at 40°C, the enzyme showed 50% reduction in activity after 96 h [21]. Naringinase from *Penicillum decumbens* revealed highest activity at 70°C in free form while in immobilized form showed highest activity at 50°C [41]. Production, purification and characterization of naringinase from *Bacillus subtilis* strain BSnari has been reported [23]. The enzyme was assessed for debittering of citrus juice. Authors obtained optimal debittering (33-36% reduction in bitterness) at 40-50°C after 4 h of treatment. Crude naringinase from *Aspergillus flavus* was characterized by Srikantha et al. [42]. Naringinase showed highest enzyme activity at pH 4.5. The enzyme was stable in temperature range of 40-45°C. The V_{max} value of the crude enzyme "naringinase" was found to be 4.8076 µmol/min. Further, the "Michaelis constant" for naringin was obtained as 4.347 g/L. Among the assessed metal ions, Hg^{2+}, Cu^{2+} and Ba^{2+} lead to decrease in activity of naringinase while, Na^{+}, Mn^{2+}, Ca^{2+} and Zn^{2+} resulted in increased activity of naringinase. González-Vázquez et al. [43] reported production of naringinase from *Aspergillus niger* ATCC1015. The V_{max} value as 13.24 U mg^{-1} and K_m value as 1.6 mM was reported by authors for the naringinase from *Aspergillus niger* ATCC1015 [43]. Purification of a novel naringinase from *Aspergillus oryzae* 11250 has been discussed earlier [44]. The optimum pH of naringinase was reported as 5.0 and temperature was 45°C. The K_m and V_{max} values of naringinase for naringin were found to be 1.60 ± 0.13 mM & 126.21 ± 5.52 µmol/(min mg), respectively.

CONCLUSION AND FUTURE PERSPECTIVES

Naringinase is an enzyme capable of hydrolysing the flavonoid naringin. Naringin is a flavonoid present in citrus fruits. This flavonoid is known to contribute to the bitterness of citrus juices. Naringinase is an important microbial enzyme having immense industrial potential. Production of this enzyme from both bacterial as well as fungal sources have been reported. This enzyme may provide alternate to the chemical processing of citrus juices. The enzyme needs to be investigated more extensively with higher pace. In current review, purification and characterization of microbial naringinase has been discussed for potent application in hydrolysing the naringin. This review may provide important information to the researchers working in the area of naringinase.

CONFLICTS OF INTERESTS

The authors declare do not declare any conflict of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not Applicable

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