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**Faculty of Food Science**

# Naringinase from probiotic bacteria and its application in production of probiotic citrus juices

**Ph.D. Thesis**

**Tran Thi Mai Anh**

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# Abbreviations

| | |
|---|---|
| β-CD | β-Cyclodextrin |
| ABTS | 2, 2’-azino-bis-(3-ethylenzthiazoline-6-sulfonic acid) |
| ANOVA | Analysis of variance |
| CCD | Central composite design |
| DNS | 2,4-dinitrosalicylic acid |
| EMP | Embden-Meyerhof pathway |
| FRAP | Ferric-reducing antioxidant power |
| GH | Glycoside hydrolase |
| HPLC | High-performance liquid chromatography |
| LAB | Lactic acid bacteria |
| PKP | Phosphoketolase pathway |
| PVA | Polyvinyl alcohol |
| RSM | Response surface methodology |
| SD | Standard deviation |
| TPC | Total phenolic content |
| TPTZ | 2,4,6-tri[2-pyridyl]-s-triazine |

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# 1. INTRODUCTION AND OUTLINE

## 1.1. Introduction

Citrus family fruits such as grapefruit, orange, limon, tangerine, etc. are typical fruits growing in tropical and subtropical regions including Vietnam. Nutritionally, these fruits are valuable as they are rich in vitamins (especially vitamin C) and antioxidants, but unfortunately, they contain high amounts of bitter compounds. Two main types of bitterness, caused by two different types of compounds, occur in citrus fruits. Flavanone neohesperidosides, as naringin in grapefruit and neohesperidin in sour oranges, provide the typical bitterness of fruits and juices from these species. The other type of bitterness, which constitutes an extremely negative quality factor in some orange juices, is produced by limonin, a triterpene derivative of the limonoid group. Limonin bitterness is known as 'delayed bitterness', since it is not detected in fresh fruits or freshly extracted juices but is developed during juice storage or by heat treatment. In general, fresh fruits do not contain limonin, but a nonbitter precursor, which converts into limonin after juice preparation. Limonin is detected by taste at concentrations of about 6–8 mg/L in orange juice (Izquierdo & Sendra, 2003). Naringin, 4',5,7-trihydroxyflavonone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside, is known as the principal component that causes the bitterness in grapefruit. Its amount varies among parts of fruit, one of the main parts containing naringin is the albedo, the fruit membrane (Yusof *et al.*, 1990; Puri & Banerjee, 2000; Thammawat *et al.*, 2008; Raithore *et al.*, 2016). It has been reported that when naringin is present in water solution in concentrations higher than 20 μg/mL, the bitter taste can be detected, however, in grapefruit juices, it is only detectable in concentrations higher than 300–400 μg/mL (Soares & Hotchkiss, 1998a). Thus, debittering process should be investigated to make these juices to be acceptable by consumers.

Reduction of bitterness has been attempted by many methods, involving changes in cultivation practices (rootstock, fertilization) and juice treatments. Debittering of processed juices seems to be the most promising approach, and some citrus industries are already equipped with debittering devices. Some techniques have been studied and developed for reducing the bitterness in citrus fruit juice, such as using of adsorbents (Chandler *et al.*, 1968; Chandler & Johnson, 1977; Barmore *et al.*, 1986; Mishra & Kar, 2003; Jungsakulrujirek & Noomhorm, 2004) or β-cyclodextrin (Chatjigakis *et al.*, 1992; Mongkolkul *et al.*, 2006), by blanching (Zid *et al.*, 2015; Jagannath & Kumar, 2016), or using chemicals to remove bitterness (Pichaiyongvongdee & Haruenkit, 2011). These techniques are classified as physicochemical methods, and they have some limitations on the quality of citrus fruit juice (removal of nutrients, flavor, color, causing turbidity, etc.) leading to unacceptability by consumers. To overcome these limitations,

biotechnological methods using enzymatic technology in fruit juice processing should be developed and applied.

Naringinase is an enzyme complex with α-L-rhamnosidase (E.C. 3.2.1.40) and β-D-glucosidase (E.C. 3.2.1.21) activities (Puri *et al.*, 2011b). This enzyme preparation is commercially attractive due to its potential usefulness in pharmaceutical and food industries. Meanwhile, α-L-rhamnosidase cleaves terminal α-L-rhamnose specifically from a large number of natural products including naringin, rutin, quercitrin, hesperidin, diosgene, terpenyl glycosides, and many other natural glycosides, whereas the β-D-glucosidase can further hydrolyze glucose molecule from some intermediers such as prunin to produce naringenin. These molecules have a great potential, especially in the food and pharmaceutical industries, due to their recognized antioxidant, anti-inflammatory, anti-ulcer, and hypocholesterolemic effects, whereas naringenin has also shown anti-mutagenic and neuroprotective activities, while prunin has antiviral activity (Lee *et al.*, 2001; Ribeiro *et al.*, 2008; Amaro *et al.*, 2009). Moreover, naringinase is commercially used in debittering and clearance of citrus fruit juices as well as enhancement of wine aromas in the food industry. While this enzyme is widely distributed in fungi, its production from bacterial sources is less commonly known. Bioinformatical analysis of genomic data of lactic acid bacteria and bifidobacteria resulted both α-L-rhamnosidase and β-D-glucosidase coding genes, thus they should synthesize naringinase enzyme (Avila *et al.*, 2009; Beekwilder *et al.*, 2009).

Due to historical and technological reasons most of the probiotic foods are based on dairy products. Unfortunately, it may cause inconveniences for some segments of consumers who do not tolerate lactose (lactose intolerance), are allergic to proteins, or simply being vegetarian. Since fruits and vegetables already contain beneficial nutrients such as minerals, vitamins, dietary fibers and antioxidants, while lacking dairy allergens, they may serve as ideal food matrices for carrying probiotic bacteria. Furthermore, fruit juices have pleasing taste profiles to all age groups, and they are perceived as being healthy and refreshing. Thus, the development of new non-dairy probiotic food products may be very much challenging, as they have to meet the consumer's expectancy for health. In this sense, many studies are carried out to develop novel probiotic fruit or vegetable products mainly focusing on soymilk, carrot juice, noni juice, pineapple, etc., but less on other tropical juices. Probiotic bacteria are generally applied in production of fermented functional foods, thus using these bacteria with high naringinase activity for fermentation of citrus juices should have high scientific and innovative impact.

## 1.2. Outline of dissertation

Recently, applications of naringinase from microbial sources to debitter citrus fruit juice, especially grapefruit juice, are more explored (Ni *et al.*, 2014; De Silva *et al.*, 2017; Pandove *et al.*, 2017; Zhu *et al.*, 2017a; Zhu *et al.*, 2017b). The various drawbacks when using chemical or physical methods for reducing the bitterness in citrus fruit juice are: (1) the juice must be previously deoiled; (2) the organoleptic properties and quality of juice may be affected by alkali solutions needed for regeneration of the adsorption columns; (3) using chemicals to remove the bitterness could alter the composition of juice or remove nutrients, flavor, color characteristic of citrus juice; (4) the chemicals used in certain cases cannot be recycled (Puri & Banerjee, 2000). These limitations could be overcome by applying biotechnological methods. It means treating the juice with enzyme naringinase in citrus fruit juice processing, as it is a viable source, has remarkable reusability, and has a less toxic effect on the environment. Keeping all this in view, the present work has been carried out under the following objectives:

- Screening probiotic strains for naringinase production
- Study of the factors that influence production of naringinase during fermentation
- Optimization of some factors for enhancement of production of naringinase by probiotic bacteria
- Characterization of crude naringinase
- Application of whole cells of probiotic bacteria for producing probiotic beverage and debittering of grapefruit juice by mono and mixed cultures

# 2. LITERATURE REVIEW

## 2.1. Citrus fruits

### 2.1.1. General introduction

Citrus is a genus of flowering trees and shrubs in the rue family, *Rutaceae*. Citrus fruits are native to southeastern Asia and are among the oldest fruit crops domesticated by humans. They are widely grown in all suitable subtropical and tropical climates and are consumed worldwide. Plants in the genus produce citrus fruits including important crops like orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), tangerine (*Citrus tangerina*), clementine (*Citrus reticulata*), kinnow (*Citrus nobilis* × *Citrus deliciosa*), grapefruit (*Citrus paradisi*), pomelo (*Citrus maxima* or *Citrus grandis*), lemon (*Citrus limon*) and lime (**Fig. 2.1**). These are consumed freshly, as juices, and utilized in processed products.

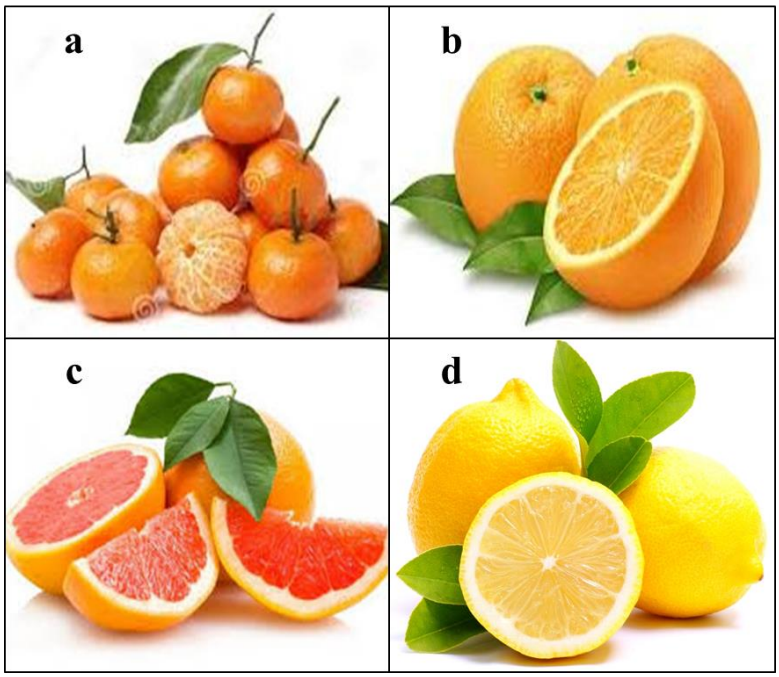


**Figure 2.1 Some kinds of citrus fruit (a)-mandarin, (b)-orange, (c)-grapefruit, (d)-lemon**

Citrus fruits have been cultured for over 4000 years. It is believed that the true citrus fruits originate from Southeast Asia. Citrus fruits are one of the largest fruit crops in the world. Total world production of citrus fruits in the 2015-2016 season was in excess of 124 million tons (FAO, 2017). The leading variety is oranges (53.9%), followed by tangerines (22%), lemons and limes (12.86%) and grapefruit (6.7%). Oranges provide the major portion in citrus fruit processing (78.4%) (**Fig. 2.2**).

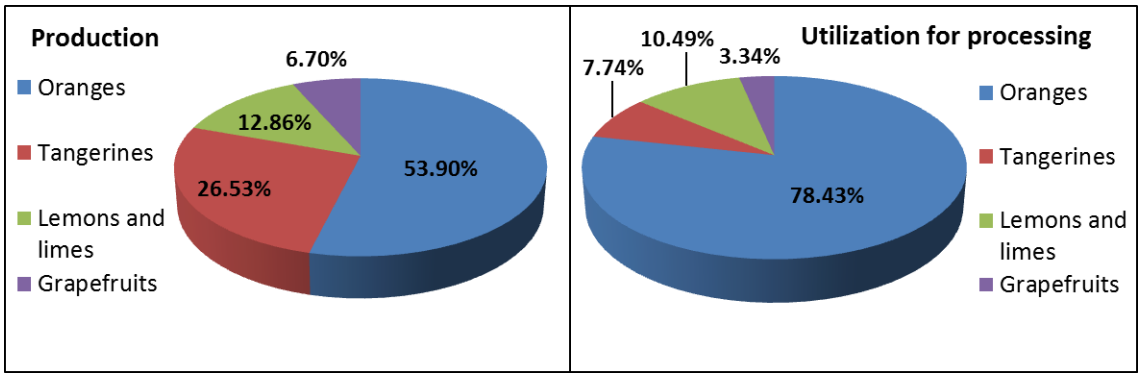


**Figure 2.2 Total world production and utilization for processing of citrus fruits in 2015/2016 season (FAO, 2017)**

In the past, citrus fruit was consumed exclusively as fresh fruit, even in countries not producing citrus. This was made possible because of postharvest stability of citrus fruit trade and the fact that in most variety of citrus, the fruit can be preserved by leaving it on the tree for a long time after maturation without spoilage. However, as the acreage of plantations and the size of the crops increased steadily, industrialization of citrus fruits became a necessity. Besides, the development of technology had a very strong impact on food industry, including citrus processing. Citrus concentrates or citrus based soft drinks such as lemonades and orangeades became the leading kind of bottled fruit beverages. About 20% of citrus fruits are processed to obtain various products, mainly juice (FAO, 2017). The most commercially important varieties include oranges, grapefruits, lemons, tangerines. Oranges account for the greatest value in terms production as well as processing, followed by grapefruits, lemons, and tangerines.

### 2.1.2. Nutritional value

Fruit juice is consumed frequently by a large portion of consumer population all over the world because of being considered as a healthy food product. Citrus fruits contain a range of key nutrients such as vitamin C, vitamin A, carotenes of various kinds (β-carotene, lutein, zeaxanthin), folate, and fiber, as well as very many non-nutrient phytochemicals including classes such as flavonoids, glucarates, coumarins, monoterpenes, triterpenes, and phenolic acids, and individual components such as hesperidin, naringin, tangeritin limonene, nomilin, perillylalcohol myrecetin, quercetin, sinsensetin, tangeretin and nobiliten. Therefore, citrus fruits play important role in production of juice beverages. Recently, the demand and market for citrus fruit juices as well as grapefruit juices are relatively high due to their significant nutritional value. The health benefits of grapefruit juices have been attributed in part to the presence of ascorbic acid (vitamin C; 33 mg/100 g), flavonoids, limonoids, coumarins and essential vitamins as folates (30 µg), niacin (0.282 mg), panthothenic acid (0.25 mg), pyridoxine (0.060 mg), riboflavin (0.020 mg), thiamine

(0.1 mg), vitamin A (33 IU), vitamin E (0.13 mg) per 100 g (USDA National Nutrient Database, 2009). Grapefruit also contain electrolytes; sodium (3-4 mg/mL), potassium (168.5 mg in 100 ml juice); minerals: calcium (12 mg), copper (39 mg), iron (0.1 mg), magnesium (9 mg), manganese (0.024 mg), zinc (0.8 mg), and β-carotenoides (14 μg) per 100 g (USDA National Nutrient Database 2009). A total of 150 g edible portion of orange provides 0.3 g fiber and 17 g of carbohydrates that can supply up to 73 kilocalories. Furthermore, fruit juices generally do not have any dairy allergens such as lactose, milk protein (Luckow & Delahunty, 2004).

**Figure 2.3 Some cultivars of grapefruit in Vietnam**

Grapefruit juice (**Fig. 2.3**) is considered as the most important nutrient food, containing a range of important nutrients for human health, like bioactive compounds such as minerals, vitamins, folate, fiber and antioxidants (Zhang, 2007), as well as containing many phytochemical compounds including flavonoids, carotenoids, glucarates, coumarins, terpenes and limonoids. These components provide many health benefits, such as antioxidant, anti-inflammatory, antimicrobial and anti-tumor activity, as well as cardiovascular protective effect, neuroprotective effects, and also inhibiting formulation of blood clots (Peterson *et al.*, 2006; Vanamala *et al.*, 2006; Kabra *et al.*, 2012; Zou *et al.*, 2016). Naringin is one of the flavonoids that presences large amounts in grapefruit and grapefruit juice. It is associated with many health benefits, mainly the naringin aglycone (naringenin). Naringin and naringenin could act as free radical scavengers and antioxidants, reduce total cholesterol level and enhance ethanol metabolism, reduce the risk of atherosclerosis, protect plasma vitamin E levels, increase bone cell activity, stimulate DNA repair in prostate cancer cells, and reduce oxidative stress and inflammatory response (Nagem *et al.*,

2001; Jeon *et al.*, 2002; Naderi *et al.*, 2003; Seo *et al.*, 2003; Gao *et al.*, 2006; Wong & Rabie, 2006; Amaro *et al.*, 2009). Naringin significantly enhances the immune system's effectiveness to avoid injury or disease of internal organs and tissues caused by oxidation by increasing the activity of catalase.

## 2.2. Flavonoids concentrations of grapefruit juices

Bioactive compounds in citrus fruits and juices have an important role in human nutrition. These include antioxidants such as ascorbic acid, flavonoids and phenolic compounds (Ghasemi *et al.*, 2009). Flavonoids are low molecular weight compounds composed of a three-ring structure with various substitutions. The basic of flavonoids' structure is comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring (C) (Middleton *et al.*, 2000). Six subclasses of flavonoids are classified based on the difference of chemical structure of the heterocyclic ring C: flavones, flavonols, flavanones, flavanols (catechins), anthocyanidins, and isoflavones (**Fig. 2.4**). Flavanones, flavones and flavonols are three types of flavonoids that occur in citrus fruits. In which, flavanones are the dominant flavonoids in citrus fruits (e.g., 98% in grapefruits, 90% in lemons and 96% in limes) (Peterson *et al.*, 2006). Hesperidine, narirutin, naringin and eriocitrin were found as the main flavonoids in citrus species (Mouly *et al.*, 1994).

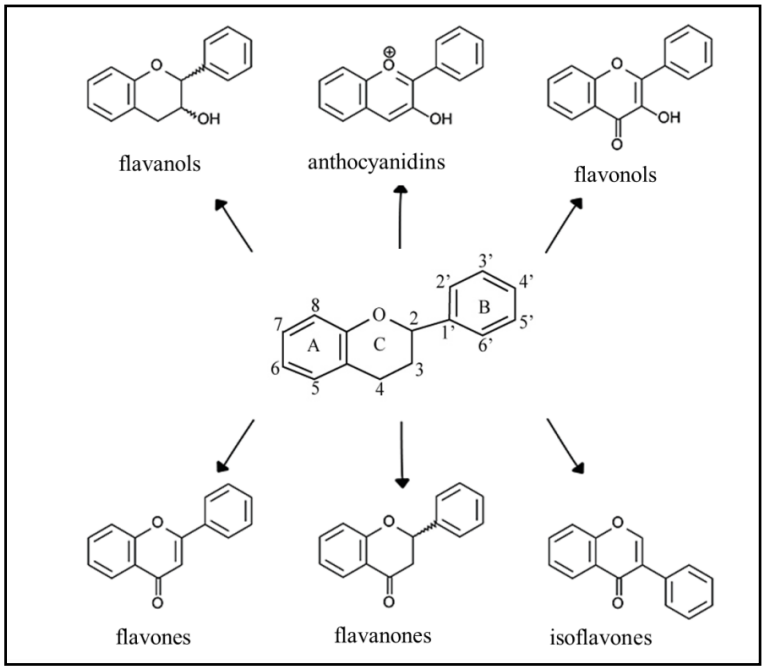


**Figure 2.4 Chemical structures of subclasses of flavonoids (Zhang, 2007)**

The concentrations of four flavanone glycosides in five grapefruit cultivars in Florida were determined by HPLC (Rouseff *et al.*, 1987). Juice samples were prepared by hand-squeezing the

grapefruit harvested from the Florida Citrus Arboretum in Winter Haven, FL. The results showed that grapefruit juices from different cultivars contained all four flavanone glycosides of naringin, narirutin, hesperidin and neohesperidin with the naringin as the predominant flavanone glycoside. The data also revealed that the commercial canned grapefruit juices contained from 2-4 times higher flavonoid concentrations than hand-squeezed juices. The explanation of the results could be that the peel and segment membranes contain higher concentrations of flavanones, which will be more extracted into the juice as the fruit is squeezed harder than the ones squeezed by hand.

Ross *et al.* (2000) analyzed nine commercial brands of grapefruit juice for their flavonoids content by HPLC. All grapefruit juices examined had the presence of flavonoid glycosides narirutin, naringin, hesperidin, neohesperidin, didymin and poncirin. Naringin (14.56 to 63.6 mg/100 mL) was found to be the major flavonoid followed by narirutin (2.25 to 12.2 mg/100 mL) and hesperidin (0.24 to 3.12 mg/100 mL) (Ross *et al.*, 2000)

In another study conducted by Vanamala *et al.* (2006), five not-from-concentrate grapefruit juices available in the US market were analyzed for their flavonoids content by HPLC. Naringin, narirutin and porcirin were the main flavonoids in all brands of grapefruit juices investigated. Naringin and narirutin content in grapefruit juices ranged from 23.5 to 37.2 mg/100 mL and from 9.1 to 11.7 mg/100 mL, respectively (Vanamala *et al.*, 2006). The concentration of individual flavonoids are summarized in **Table 2.1.**

**Table 2.1 Flavonoids concentration of commercial grapefruit juices from different studies**

| Flavonoids | Mean mg/ 100 mL | Range mg/ 100 mL | References |
|---|---|---|---|
| Naringin | 35.35 | 0.48 – 119.7 | 1,2,3,4,5 |
| Narirutin | 8.84 | 2.6 - 12.4 | 2,3,4 |
| Naringenin | 2.01 | 0 – 16.2 | 1,2,5 |
| Poncirin | 1.06 | 0.12 – 2.36 | 2,4 |
| Hesperidin | 1.76 | 0.24 - 3.12 | 2,3 |
| Didymin | 0.83 | 0.04 - 1.72 | 2 |
| Neohesperidin | 0.72 | 0.04 - 1.12 | 2,3,4 |
| Quercetin | 0.6 | 0.2 - 0.88 | 2,4 |

[1]Ho *et al.* (2000), [2]Ross *et al.* (2000), [3]Rouseff *et al.* (1980), [4]Vanamala *et al.* (2006), [5]Wanwimolruk *et al.* (2006)

### 2.2.1. Bitterness

The processing of citrus juice faces problems in terms of "bitterness" and "delayed bitterness" (Puri *et al.*, 1996b). Bitterness in citrus fruits is primarily related to two compounds, flavonoids (e.g. naringin) and limonoids (e.g. limonin). Naringin is the most abundant flavonoid in grapefruit juice, followed by narirutin, quercetin and naringenin (Mansell *et al.*, 1983; Ross *et al.*, 2000; Vanamala *et al.*, 2006; Igual *et al.*, 2011). The presence of the bitterness in citrus fruit juices is an undesirable quality for juice and beverage industry. It is the major hurdle to the commercial acceptance of citrus juice (Narnoliya & Jadaun, 2019).

Naringin and limonin are identified as major contributors for "immediate" and "delayed" bitterness, respectively (Puri *et al.*, 2005; Narnoliya & Jadaun, 2019). Limonin bitterness is known as "delayed bitterness", because it is not detected in fresh fruits or freshly extracted juices, but developed during storage or heat treatment. Generally, fresh fruits do not contain limonin, because it is formed from a non-bitter precursor after juice preparation (Izquierdo & Sendra, 2003). Its taste threshold is approximately 6-8 mg/L in orange juice. While naringin is the predominant bittering agent in grapefruit (*Citrus paradisi*) and pomelo (*Citrus maxima*, *Citrus grandis*), whereas neohesperidin is a major factor for bitterness in sour orange (*Citrus sinensis*), although neoeriocitrin and poncirin are present in minor concentrations in citrus juices, as well (Kawaii *et al.*, 1999).

Naringin is known as 4',5,7-trihydroxyflavonone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside. Its concentration varies among parts of fruit such as peel, seed and flesh. One of the main parts containing naringin is the albedo and the fruit membrane. When it is squeezed, the naringin is extracted into the juice. In the study of Yusof *et al.* (1990), naringin content in citrus fruits were determined by HPLC. The results showed that pomelo had higher naringin content than rough lime. The highest proportion of naringin was found in peel (3910 μg/g) compared to the juice (220 μg/g) (Yusof *et al.*, 1990). The peel, which represents almost one half of the citrus fruit mass, contains the highest concentrations of flavonoids (Anagnostopoulou *et al.*, 2006). The naringin can be detected by taste at the concentration of about 20 ppm in water. All processed grapefruit juice contains naringin above 50 ppm (Puri & Banerjee, 2000), so it is easy to taste the bitterness in grapefruit juice. The content of naringin in sweet pomelo (*Citrus grandis* (L) Osbeck) can reach 11.9 mg/100 g (Zhou, 2012).

In grapefruit the naringin concentration varies from cultivar to cultivar. Rouseff *et al.* (1987) reported that the content of naringin in five different cultivars of grapefruit (*Citrus paradise*): Ducan, Foster, Marsh, Ruby Red and Starr Ruby were 197 ppm, 133 ppm, 152 ppm, 124 ppm and 73 ppm, respectively. In the study of Pichaiyong and Haruenkit (2009), seven pomelo

cultivars grown in Thailand were analyzed for the distribution of limonin and naringin in different parts of pummel. The highest concentration of limonin was observed in the seeds of all cultivars ranging from 1375.31-2615.3 ppm, followed by the albedo (135.2-352.72 ppm), flavedo (130.16-295.49 ppm), segment membranes (85.81-293.14 ppm), and juice (10.07 – 29.62 ppm). Naringin was found in a higher amount than limonin in all fruit parts of the cultivars studied. The naringin content of fruit parts in a decreasing order was albedo (10065.06-28508.01 ppm) > flavedo (2483.96-8964.24 ppm) > segment membranes (1799.48-4369.5 ppm) > seeds (257.87-426.66 ppm) > juice (242.63-386.45 ppm) (Pichaiyongvongdee & Haruenkit, 2009).

Bitterness can be acceptable up to a certain extent, but the excessive bitterness is undesirable to the consumers. Although it is abundant in immature fruit, its concentration decreases during fruit ripens. The variation of limonin and naringin contents in grapefruit (*Citrus paradise*) peel albedo was observed over three seasons in Marsh grapefruit (Shaw *et al.*, 1991). The observations were similar for both naringin and limonin in all three seasons. More 10-fold decrease in limonin content in albedo was observed during maturing of three seasons, 1985-1986, 1986-1987, and 1987-1988 season, from 162 to 21 ppm, 89 to 15 ppm and 221 to 13 ppm, respectively. Naringin content in albedo showed a relative decrease with maturity: 24%, 17% and 29%. These results were in agreement with the study of Del Rio *et al.* (1997). The highest amount of naringin was found in the immature grapefruit. The concentration of naringin in a whole immature grapefruit (3-7 mm diameter) was 37.8 g/100 g of dry tissue compared to 7.2 g/100 g dry tissue for a mature fruit (70-80 mm diameter). In addition, other flavonoids such as hesperidin and neohesperidin were also in higher concentrations in immature grapefruit than in mature fruit (Del Rio *et al.*, 1997).

Generally, in order to limit the bitterness going into the citrus fruit juices, the fruit maturity should be considered when processing. Furthermore, the pressure used in squeezing the citrus fruit juices should be lowered to minimize the extraction of the bitterness from the albedo, flavedo, and segment membrane into the juice.

### 2.2.2. Debittering technology

The reduction of bitterness is necessary to control the quality and improve the commercial value of grapefruit juices as well as to increase their acceptance by consumers (Ribeiro & Ribeiro, 2008). The bitter taste can be detected when naringin is present in water solution in concentrations higher than 20 μg/mL, however, in grapefruit juices, it is only detectable in concentration higher than 300–400 μg/mL (Soares & Hotchkiss, 1998a). In order to debitter, some techniques have been investigated.

### 2.2.2.1. Physical methods

- **Adsorption**

In the past, several adsorptive materials including cellulose acetate and its different derivatives such as cellulose acetate butyrate, cellulose triacetate, cellulose esters and Florisil (activated magnesium silicate), have been explored successfully for debittering in different citrus fruits (Chandler *et al.*, 1968; Chandler & Johnson, 1977; Barmore *et al.*, 1986; Tsen & Yu, 1991). These adsorbents can be used individually or in combination with others. The efficiency and suitability of various adsorbents vary according to their physical properties. Other polymers like polyvinylpyrrolidone, nylon polymers, synthetic neutral resins (Amberlite XAD-2, XAD-4, XAD-7, and XAD-16 or Amberlite IR 120, IR400), diatomaceous earth, and granulate of activated carbon were also studied for the debittering process (Johnson & Chandler, 1982; Nisperos & Robertson, 1982; Ribeiro *et al.*, 2002; Mishra & Kar, 2003; Jungsakulrujirek & Noomhorm, 2004).

- **Blanching**

Blanching is a process of heat treatment, applied in fruit and vegetable industry for deactivating the endogenous enzymes, which affect their organoleptic and nutritional values as well as shelf life (Fellows, 2009). While water blanching was able to remove about 38% and 48% of bitter flavanones when treated at 95 °C and 85 °C temperature, respectively, whereas steam blanching revealed a good retention of bitterness (Zid *et al.*, 2015). Recently, blanching was applied on Nagpur (*Citrus reticulata Blanco*), Kinnow (*Citrus nobilis* × *Citrus deliciosa*), and Mandarin (*Citrus reticulata*) fruit at a mild temperature of 65 ºC, which was followed by osmodehydration. It was observed that naringin content was reduced to 50% and further decrease was observed to 3-100 mg/100 g during storage time (Jagannath & Kumar, 2016).

### 2.2.2.2. Chemical methods

β-cyclodextrin (β-CD) (Cycloheptaamylose) is a cyclic oligosaccharide, which is made up of seven α-1,4-linked D-glucopyranose units in a cyclic manner. It is soluble in water, sweet in taste (Chatjigakis *et al.*, 1992), and produced by the enzymatic conversion of starch. β-CD is usually used in pharmaceutical, food and nutraceutical, cosmetic, agricultural, and chemical industries due to being cheaper than α and γ forms (Del Valle, 2004). For the debittering of juice extracted from citrus fruits (citrus natsudaidai grapefruit, and iyo orange), 0.3% β-CD (w/w) was used, which resulted in the decrease of bitterness to an acceptable level (Konno *et al.*, 1981). In a study on reducing the bitterness of Tangerine *Citrus Reticulata* Blanco juice by β-CD, bitterness

decrease by the batch and column processes were 70 and 94% when using 3% β-CD at room temperature, respectively (Mongkolkul *et al.*, 2006).

Some chemicals showed effective bitterness suppressing activity such as neodiosmin, sucrose and citric acid. Sucrose, citric acid, and combinations of these constituents in a model system increased the threshold of limonin and naringin several fold (Guadagni *et al.*, 1973). In another work of these authors, sucrose, hesperetin dihydrochalcone glucoside and neohesperidin dihydrochalcone were effective in suppressing naringin bitterness (Guadagni *et al.*, 1974). The addition of 60-100 ppm of neodiosmin to orange juice containing 10 ppm of added limonin, reduced limonin bitterness to the equivalent of 4-5 ppm of limonin in orange juice (Guadagni *et al.*, 1976).

Ethylene was applied in pomelo fruit (C. *grandis* L. Osbeck) for monitoring the content of limonin. Treatment with ethylene at 200 ppm concentration for 1.30 h exposure led to a decrease in limonin content by 78.38% and a slight decrease in naringin content. This method had no effect on antioxidant capacity or on nomilin, eriocitrin and neoeriocitrin concentrations of the juice (Pichaiyongvongdee & Haruenkit, 2011).

The physical and chemical methods have several limitations, so they are not applied at industrial scale. First, the methods could alter the composition of the juices, as well as removal of nutrients, flavor and color occurs through chemical reactions during process. This drawback affects to the acceptability by consumers. Second, the cost of the chemicals is also a limitation. Moreover, the used chemicals cannot be recycled. Sometimes, these chemicals have undesirable impact on the environment due to their hazardous nature and lack of a proper channel for disposal. Lastly, these processes are not viable technologies for applying at large scale, so researchers are trying to develop more sustainable and eco-friendly alternatives for the debittering of fruit juices (Puri *et al.*, 1996b; Narnoliya & Jadaun, 2019).

### 2.2.2.3. Biotechnological methods

Limitations of physicochemical processes can be overcome by introducing the biotechnological methods in fruit juice processing by using naringinase or using of whole microbial cells with the ability to produce naringinase. A large number of microorganisms were screened for reducing the bitterness of citrus fruit as well as decreasing the naringin in grapefruit juice. This problem can be solved by microbes with the ability of naringinase production. Lots of study focused on production of naringinase from *Aspergillus* species such as *Aspergillus niger* (Kishi, 1955; Bram & Solomons, 1965; Puri & Kalra, 2005), *Aspergillus kawachi* (Koseki *et al.*, 2008), or *Aspergillus oryzae* (Zhu *et al.*, 2017a). Subsequently, other species were also recognized

as good producers of naringinase enzyme such as *Penicillium decumbens* (Norouzian *et al.*, 2000) and *Penicillium ulaisen* (Rajal *et al.*, 2009). Recently, there are more publications on naringinase from bacteria (Michlmayr *et al.*, 2011; Pavithra *et al.*, 2012; Kumar *et al.*, 2015; Zhu *et al.*, 2017b). Further information on naringinase as well as biotechnological methods for debittering of citrus fruit juice will be mentioned in detail.

## 2.3. Naringinase

### 2.3.1. General information

Naringinase is an enzyme complex with α-L-rhamnosidase (EC.3.2.1.40) and β-D-glucosidase (EC.3.2.1.21) activities. Naringinase occurs widely in nature and has been found in plants, fungi and bacteria (Ribeiro, 2011). Naringin can be hydrolyzed by α-L-rhamnosidase releasing prunin and rhamnose (**Fig. 2.5**).

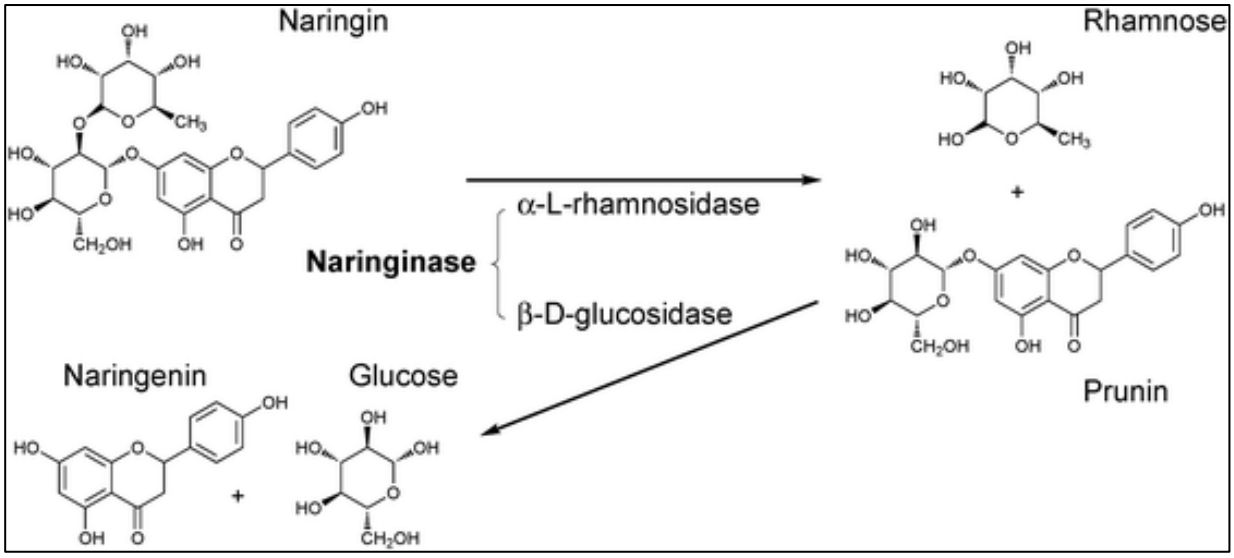


**Figure 2.5 Hydrolysis of naringin into rhamnose, prunin, glucose and naringenin by naringinase**

Then β-D-glucosidase hydrolyses prunin into non-bitter naringenin (4,5,7-trihydroxy flavanone) and glucose (Puri *et al.*, 2011b). Products formed by the hydrolysis reaction of naringinase have a great potential, especially in the food and pharmaceutical industries. They are recognized as antioxidant, anti-inflammatory, anti-ulcer and hypocholesterolemic agents (Lee *et al.*, 2001; Ribeiro *et al.*, 2008; Amaro *et al.*, 2009). According to Bok *et al.* (2000), naringin and its hydrolyzed product naringenin can inhibit the activity of acyl CoA-cholesterol-o-acyltransferase, prevent or treat hepatic diseases by inhibiting the accumulation of macrophage-lipid complex. Furthermore, naringenin has also shown anti-mutagenic and neuroprotective

activities, while prunin has antiviral activity. Therefore, the hydrolyzed components produced by naringinase activity can be used as compositions or process materials in production of pharmaceutics, cosmetic and food. Recently, naringinase is commercially used in debittering and clearance of citrus fruit juices, as well as enhancement of wine aromas in food applications (Thomas *et al.*, 1958).

### 2.3.2. Sources

Although microorganism has been reported being the main sources for naringinase production, these enzymes had been first found from plant source, in 1938 from celery seeds (Hall, 1938), then from grapefruit leaves (Thomas *et al.*, 1958) and from buckwheat (Bourbouze *et al.*, 1976). Pig liver is the only mammalian source of naringinase found until now (Qian *et al.*, 2005). Producing naringinase from fungi has been documented thoroughly, but only a few studies are found in the literature on naringinase from bacteria.

**Table 2.2 Different sources for naringinase production**

| Source | Microorganism | References |
|---|---|---|
| Plant | Celery seeds *(Apium graveolens)* | (Hall, 1938) |
| | Grapefruit leaves | (Ting, 1958) |
| | *Rhamnus daurica* | (Suzuki, 1962) |
| | Buckwheat *(Fagopyrum esculentum)* | (Bourbouze *et al.*, 1976) |
| Gastropod | *Turbo cornutus* | (Kurosawa *et al.*, 1973) |
| Mammal | Pig liver | (Qian *et al.*, 2005) |
| Fungi | *Aspergillus niger* | (Bram & Solomons, 1965) |
| | *Penicillium decumbens* | (Young *et al.*, 1989) |
| | *Rhizopus nigricans* | (Shanmugam & Yadav, 1995) |
| | *Aspergillus niger* | (Manzanares *et al.*, 1997) |
| | *Aspergillus nidulas* | (Orejas *et al.*, 1999b) |
| | *Penicillium decumbens* PTCC5248 | (Norouzian *et al.*, 2000) |
| | *Aspergillus aculeatus* | (Manzanares *et al.*, 2001) |
| | *Aspergillus terrus* | (Gallego *et al.*, 2001) |
| | *Aspergillus niger* MTCC1344 | (Puri *et al.*, 2005) |
| | *Aspergillus niger* CECT2008 | (Busto *et al.*, 2007) |
| | *Aspergillus kawachii* | (Koseki *et al.*, 2008) |
| | *Penicillium decumbens* | (Mamma *et al.*, 2004) |
| | *Aspergillus niger* BCC 25166 | (Thammawat *et al.*, 2008) |

| Source | Microorganism | References |
|---|---|---|
| | *Penicillium ulaiense* | (Rajal *et al.*, 2009) |
| | *Aspergillus sojae* | (Chang *et al.*, 2011) |
| | *Aspergillus niger* VB07 | (Kumar *et al.*, 2010) |
| | *Aspergillus oryzae* 1125 | (Zhu *et al.*, 2017a) |
| Yeast | *Hanshula anomala*<br>*Debaryomyces polymorphus* | (McMahon *et al.*, 1999) |
| | *Pichia angusta* X349 | (Yanai & Sato, 2000) |
| | *Cryptococcus laurentii* | (Lei *et al.*, 2011) |
| Bacteria | *Bacteriodes distasonis,* JY-1 | (Jang & Kim, 1996) |
| | *Thermomicrobium roseum* | (Jang & Kim, 1996) |
| | *Bacillus sp.* GL1 | (Hashimoto *et al.*, 1999) |
| | *Pseudomonas paucimobilis* | (Miake *et al.*, 2000) |
| | *Clostridium stercorarium* | (Zverlov *et al.*, 2000) |
| | *Geothermus vaporicell* | (Birgisson *et al.*, 2004) |
| | *Sphingomonas paucimobilis* | (Hashimoto & Murata, 1998); (Miyata *et al.*, 2005) |
| | *Burkholderia cenocepacia* | (Cardona *et al.*, 2006) |
| | *Ralstonia pickettii* | (Orrillo *et al.*, 2007) |
| | *Pseudoalteromonas sp.* | (Mazzaferro *et al.*, 2008) |
| | *Lactobacillus acidophilus* | (Beekwilder *et al.*, 2009) |
| | *Lactobacillus plantarum* NCC245 | (Avila *et al.*, 2009) |
| | *Staphylococcus xylosus* MAK2 | (Puri *et al.*, 2010), (Puri *et al.*, 2011a) |
| | *Pediococcus acidilactici* | (Michlmayr *et al.*, 2011) |
| | *Serratia sp.* | (Pavithra *et al.*, 2012) |
| | *Micrococcus sp.* | (Kumar *et al.*, 2015) |
| | *Bacillus amyloliquefaciens* 11568 | (Zhu *et al.*, 2017b) |

### 2.3.2.1. Fungal naringinase

One of the first reports studying naringinase production with fungi was the work of Kishi, published in 1955 (after Ribeiro, 2011). In 1958, Thomas *et al.* (1958) cultured a large number of microorganisms (about 100 strains) for collecting the crude culture extracts to obtain naringinase. Then the isolated crude enzymes were partially purified by alcohol precipitation, subsequently recovered by filtration, and dried in air. The selected enzyme preparation could rapidly hydrolyze in vitro in the pH rang of 3.5-5.0 at the temperature range of 20-50 °C. This preparation is also applied in debittering natural grapefruit juices (Thomas *et al.*, 1958).

❖ **Production naringinase from *Aspergillus* species**

*Aspergillus niger* was considered as the best producer of naringinase. Many researches focused on naringinase production from *Aspergillus* species from the past until present times (Bram & Solomons, 1965; Puri *et al.*, 2005; Busto *et al.*, 2007; Thammawat *et al.*, 2008; Kumar *et al.*, 2010). In the study of Bram and Solomons (1965), *A. niger* NRRL 72-4 was fermented submerged in the medium. The naringinase activity was higher when soya bean meal-yeast extract and corn steep liquor-yeast extract in low amounts were added into the fermentation media. The medium with malt extract supplementation did not supported naringinase production, although malt extract is a good source for the growth of microorganisms. On the contrary, the corn steep liquor supplemented medium promoted high enzyme production and was considered as an inducer factor for naringinase. Oxygen also was a factor that affected naringinase production, since the lowest stirrer speed used gave the highest enzyme activity.

The naringinase production from *A. niger* MTCC 1344 (Puri *et al.*, 2005) was affected by the carbon source, nitrogen source, growth factors, metal ions and environmental factors (pH, temperature). The highest naringinase production was obtained in the presence of rhamnose and molasses with concentration of 10 g/L after 8 days of fermentation.

The work of Busto *et al.* (2007) introduced naringinase production from *A. niger* CECT 2008 in modified Martinez-Madrid medium (without fructose and containing 100 μg/mL of naringin as sole carbon source) with initial pH of 7.

A large number of 348 fungi was isolated from 128 various host samples, collected from 11 different sources in Thailand and China, to screen for naringinase activity (Thammawat *et al.*, 2008). Forty fungal isolates were obtained from the primary screening, when all 384 fungi were cultivated at 40 °C for 7 days in 7 different media. *A. niger* BCC 25166 was selected from the forty fungal isolates for its performance in the secondary screening, when both glycosidase activities, α-L-rhamnosidase and β-D-glucosidase at 40 °C and pH 4.0, and naringinase activity at 50 °C, 55 °C and 60 °C and at both pH 3.0 and pH 4.0 were measured. The best conditions for production of enzyme had been observed in submerged fermentation (Czapek-Dox medium containing 0.1% naringin, pH 4.0) with $10^5$ spores/mL of inoculum concentration. Characteristics of the naringinase obtained were also investigated in this study. All naringinases, α-L-rhamnosidase and β-D-glucosidase had pH 4.0 as optimum. The optimum temperature of naringinase and α-L-rhamnosidase was 60 °C, whereas β-D-glucosidase showed the maximum activity in the range of 60 – 70 °C. The highest naringinase production of this fungus (117.77 U/mg protein) could be obtained by supplementing the medium with 3.75 g/L rhamnose and

2.5 g/L $NaNO_3$ as carbon and nitrogen sources. Soya peptone (2.5 g/L) could be used for high production of α-L-rhamnosidase (303.20 U/mg protein).

In another study, *A. niger* VB07 was isolated from the soil of citrus fruit market for producing extracellular naringinase in a liquid medium (Kumar *et al.*, 2010). Various factors affecting to the production of enzyme during fermentation has been examined so as to improve overall enzyme yield. Among different inducers (hesperidin, naringenin, rutin, naringin), naringin showed as the best inducer for naringinase production with 1.0% of naringin concentration. Different source of carbon, nitrogen and amino acids were also added to the medium to determine their effect on naringinase production. The influence of temperature and initial pH on growth and enzyme activity was checked. The results showed that the medium containing 0.1% of naringin, 0.5% of rhamnose, 0.25% of peptone and 10 mM of glycine was optimal for production of naringinase (17.28 IU/mL) in fermentation at 28 °C for 7 days and with the initial pH of 4.5.

The naringinase production from other *Aspergillus* species also was checked. Manzanares *et al.* (2001) purified and characterized two different α-L-rhamnosidase, RhaA and RhaB, from *Aspergillus aculeatus* (Manzanares *et al.*, 2001). Extracellular naringinase produced by *Aspergillus aculeatus* JMUdb058 was purified, identified and characterized (Chen *et al.*, 2013). The authors gave deep insight into the structure of the naringinase complex and the hydrolysis of naringin and other glycosides. The naringinase as well as its subunits, α-L-rhamnosidase and β-D-glucosidase, all had optimal activities at approximately pH 4.0 at 50 °C, and were stable between pH 3.0 and 6.0 and below 50 °C. The $K_m$ value and the $k_{cat}/K_m$ ratio of enzyme complex and its subunits were determined. Naringinase, α-L-rhamnosidase and β-D-glucosidase had $K_m$ values of 0.11 mM, 0.23 mM and 0.53 mM corresponded to $k_{cat}/K_m$ ratios of 14034 $s^{-1}$ $mM^{-1}$, 14146 $s^{-1}$ $mM^{-1}$ and 7733 $s^{-1}$ $mM^{-1}$, respectively.

α-L-rhamnosidase was produced employing *A. kawacchii* grown on L-rhamnose as the sole carbon source by Koseki *et al.* (2008). The purified enzyme exhibited optimal activity at pH 4.0 and temperature of 50 °C. Moreover, it showed good thermostability when its activity retained more than 80% of original activity following incubation at 60 °C for 1 h.

Recently, naringinase was collected and purified from *Aspergillus oryzae* 11250 cultured in the presence of orange peel (Zhu *et al.*, 2017a). The highest purified enzyme activity was obtained at temperature 45 °C and pH 5.0. This enzyme was stable at 30 °C for 5 h. The $K_m$ and $V_{max}$ of naringinase toward naringin determined by the Lineweaver-Burk method were 1.6 mM and 126.21 μmol/min*mg, respectively. The hydrolysis capability of naringinase was evaluated also with other substrates such as neohesperidin, hesperidin, and *p*-nitrophenyl-α-L-rhamno-pyranoside (Jang & Kim, 1996).

❖ **Production of naringinase from other fungus species**

*Penicillium* species are the favored sources for naringinase production. Naringinase from *Penicillium decumbens* has been studied by Nourouzian *et al.* (2000) and Mamma *et al.* (2004). The substrates naringin and rhamnose could be used as carbon sources and inducers for the naringinase production from *Penicillium decumbens* PTCC 5248 (Norouzian *et al.*, 2000), whereas rutin could stimulate production of enzyme (Mamma *et al.*, 2004). According to Norouzian *et al.* (2000), citric acid, glucose, $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ all inhibited naringinase activity. The commercial naringinase has been produced by Merck from *P. decumbens*.

The work of Rajal *et al.* (2009) revealed that *Penicillium ulaiense* is a post-harvest pathogenic fungus that attacks citrus fruits. Under this study, α-L-rhamnosidase was produced in a stirred-batch reactor using rhamnose as the main carbon source. The optimal pH and temperature of the enzyme were 5.0 and 60 °C, respectively. It was observed to be thermostable and good operational in white wine. $Co^{2+}$ had positive effect on α-L-rhamnosidase activity; EDTA, $Mn^{2+}$, $Mg^{2+}$ and $Cu^{2+}$ reduced the activity by different degree, and $Hg^{2+}$ completely inhibited the enzyme. The *p*-nitrophenyl-α-rhamnoside was evaluated to be a better inducer than naringin for enzyme production. The fungus could be considered as a potential enzyme producer in food and pharmaceutical industry, since *P. ulaiense* does not produce mycotoxins (Rajal *et al.*, 2009). The other strain, *Penicillium corylopholum* MTCC-2011, was also good producer for extracellular α-L-rhamnosidase (Yadav *et al.*, 2017).

### 2.3.2.2. Bacterial naringinase

Previously, almost studies on naringinase focused on fungi as source, while on bacterial naringinase were quite scarce. Recently, naringinase produced from bacteria have received more attention, but still only a few naringinase of bacterial origin have been reported. Some reports are also available for α-L-rhamnosidase. One was from *Bacteroides* JY-6, an intestinal bacterium of human origin (Jang & Kim, 1996). The enzyme consists of two subunits of MW 120 kDa with pI and optimal pH values of 4.2 and 7.0, respectively. The result also revealed that the enzyme activity is strongly repressed by L-rhamnose, L-fructose, saccharic acid, 1,4-lactone, *p*-chlormercuriphenylsulfonic acid and $Pb^{2+}$. The purified enzyme α-L-rhamnosidase, extracted from the cells of *Pseudomonas paucimobilis* FP2001, had a molecular weight of 112 kDa and isoelectric point of 7.1 (Miake *et al.*, 2000). The optimal pH and temperature of the enzyme were 7.8 and 45 °C, respectively. The enzyme activity was stimulated by $Ca^{2+}$ and remained stable for several months at -20 °C. Two thermostable α-L-rhamnosidase were identified from the thermophilic bacterium PRI-1686 (Birgisson *et al.*, 2004). The thermostable α-L-rhamnosidase has also been

reported from the thermophilic anaerobic bacterium *Clostridium stercorarium* (Zverlov *et al.*, 2000). In another document, cold-active α-L-rhamnosidase was found in some *Pseudoalteromonas* and *Ralstonia pickettii* species isolated from sea water and from the sub-Antarctic environment (Orrillo *et al.*, 2007). In their continuing work with intracellular α-L-rhamnosidase from *Pseudoalteromonas sp*. 005NJ, the authors revealed that the enzyme activity was enhanced threefold by D-fructose and inhibited by D-glucose, D-galactose and L-rhamnose (Mazzaferro *et al.*, 2008).

Puri *et al.* (2009) found an extracellular naringinase from *Staphylococcus xylosus* MAK2 isolated from soil. The effect of different physical and chemical parameters such as pH, temperature, agitation and inducer concentration were obtained (Puri *et al.*, 2011a). Especially, citrus peel powder prepared from dried citrus peel improved enzyme production (Puri *et al.*, 2011a). Continuing the study on naringinase, response surface methodology was used to optimize the fermentation medium for the improvement of naringinase production by *Staphylococcus xylosus* MAK2 (Puri *et al.*, 2010).

Extracellular naringinase also was found from *Serratia* sp., when Pavithra *et al.* (2012) isolated and tested for naringinase activity four strains of naringin degrading bacteria (Pavithra *et al.*, 2012). In other study, naringinase producing microorganisms were isolated from soil (Kumar *et al.*, 2010). A total of 10 isolates showed the naringinase activity. Based on morphological, microscopic and biochemical characteristics of the isolates, the bacterium with the highest activity was identified as *Micrococcus* sp. After purification, the molecular weight of the enzyme was determined to be 48 kDa.

Recently, the naringinase from a newly isolated strain of *Bacillus amyloliquefaciences* 11568 was reported (Zhu *et al.*, 2017b). The optimum pH and temperature for the naringinase and its α-L-rhamnosidase and β-D-glucosidase activities were pH 7.5 and 45 °C, respectively. For hydrolyzation reaction, between pH 3.5-8.5 and below 45 °C, the enzymes were stable. The naringinase can hydrolyze different substrates including naringin, neohesperidin and other glycosides. The first report on yeast producing naringinase is on *Williopsis californica* Jmudeb007 (Ni *et al.*, 2011).

### ❖ Naringinase from probiotics

α-L-rhamnosidase from lactic acid bacteria has been studied by (Avila *et al.*, 2009; Beekwilder *et al.*, 2009). The genome sequences of food-grade lactobacilli were screened for putative rhamnosidase (Beekwilder *et al.*, 2009). Two rhamnosidase genes ($ram1_{Lp}$ and $ram2_{Lp}$) were identified in the genome of *Lactobacillus plantarum*, while only one rhamnosidase gene

(*ram*$A_{La}$) was found in *Lactobacillus acidophilus*. Three genes were introduced into *Escherichia coli* for expression of enzymes. Then, gene products were tested for enzyme activity. $Ram1_{Lp}$, $Ram2_{Lp}$ and $RamA_{La}$ proteins were able to convert rutin and other rutinosides, while $RamA_{La}$ protein was able to split naringin. These results could be explained by the linkage specificity of these enzymes; $Ram1_{Lp}$ and $Ram2_{Lp}$ liberated 1→6 conjugated rhamnose residues, but not 1→2 conjugated rhamnose residues. $RamA_{La}$ acted on both 1→6 and 1→2 conjugated rhamnose residues, like those present in rutinosides and neohesperidosides, respectively. Both enzymes, $Ram1_{Lp}$ and $RamA_{La}$, showed the highest activity at temperatures from 37 to 45 °C, and they are inactivated at higher temperatures. The optimal pH was around 6.0 for both enzymes.

The intracellular α-L-rhamnosidase activity of *Lactobacillus plantarum* NCC245 was selected to be examined in more detail, after screening the α-L-rhamnosidase activity from 216 strain representing 37 species and eight food-grade bacteria (Avila *et al.*, 2009). The enzyme activity was inhibited by glucose and promoted by L-rhamnose. Two α-L-rhamnosidase genes also were identified in the genome of *L. plantarum* NCC245. Both enzymes showed the best ability for hydrolyzation to hesperidin and rutin.

Another probiotic bacterium expressing α-L-rhamnosidase was *Pediococcus acidilactici* DSM 20284 (Michlmayr *et al.*, 2011). In combination with a bacterial β-glucosidase, they released the monoterpenes linalool and *cis*-linalool oxide from a Muscat wine extract under ideal conditions for enhancement of wine aroma.

### 2.3.3. Molecular and structural characteristics of naringinase

Up to now, most bacterial naringinase genes studied expressed α-L-rhamnosidase. The number of genes known to encode α-L-rhamnosidase is much lower than the number of α-L-rhamnosidase that have been purified and characterized. Cloning has been performed mainly by two methods: (1) construction of a library followed by selection of clones by screening for α-L-rhamnosidase activity using substrate p-nitrophenyl-α-rhamnopyranoside or/and (2) the construction of a library followed by selection of clones by screening for α-L-rhamnosidase production with polyclonal antibodies (Manzanares *et al.*, 2007).

α-L-rhamnosidase enzymes are classified into three glycoside hydrolase (GH) families: GH28, GH78 and GH106 based on sequence similarity according to the CAZy (Carbohydrate-Active Enzymes) database (Lombard *et al.*, 2013). To date, few genes encoding bacterial GH78 α-L-rhamnosidase genes have been cloned and heterologously expressed. Most of them are from lactic acid bacteria: two genes (*rhaB1* and *rhaB2*) from *Lactobacillus plantarum* NCC245 (Avila *et al.*, 2009), two genes (*ram1*$_{Lp}$ and *ram2*$_{Lp}$) from *Lactobacillus plantarum* and *ramA*$_{La}$ from

*Lactobacillus acidophilus* (Beekwilder *et al.*, 2009), two genes (*ram* and *ram2*) from *Pediococcus acidilactici* (Michlmayr *et al.*, 2011), α-L-rhamnosidase gene from *Bifidobacterium breve* ATCC15700 (Zhang *et al.*, 2015), α-L-rhamnosidase gene from *Bifidobacterium dentitum* (Bang *et al.*, 2015). There are others from different sources: gene *ramA* from *Clostridium stercorarium* (Zverlov *et al.*, 2000), two genes (*rhaA* and *rhaB*) from *Aspergillus aculeatus* (Manzanares *et al.*, 2001), two genes (*rhaA* and *rhaB*) from *Bacillus* sp. strain GL1 (Hashimoto *et al.*, 2003), two genes (*rhmA* and *rhmB*) from a thermophilic bacterium (Birgisson *et al.*, 2004), gene *rhaM* from *Sphingomonas paucimobilis* (Miyata *et al.*, 2005), gene *rhaA* from *Aspergillus kawachii* (Koseki *et al.*, 2008), and gene BtRha78A from *Bacteroides thetaiotaomicron* VPI-5482 (Li *et al.*, 2018).

The first α-L-rhamnosidase encoding gene (*ramA*), isolated from the thermophilic anaerobic bacterium *Clostridium stercorarium (Zverlov et al., 2000)*, consists of 873 or 874 amino acids codes for a protein with a predicted molecular mass of 100435 Da. Circular dichroism spectroscopy was used to analyze global secondary structure of protein consisting of 27% α-helices and 50% β-sheets. However, the authors also revealed that the gene *ramA* from *C. stercorarium* is rather rare trait among bacteria. The sequence of the RamA protein was not similar to the other glycoside hydrolases.

In another work, the genes *rhaA* and *rhaB* for two α-L-rhamnosidases cloned from *Bacillus* sp. strain GL1 had been express in *Escherichia coli*, and the nucleotide sequences of the genes were determined (Hashimoto *et al.*, 2003). Gene *rhaA* (2661bp) contained an open reading frame (ORF) encoding a protein (RhaA: 886 amino acids) with a molecular weight (MW) of 98,280. Gene *rhaB* (2871 bp) contained an ORF encoding a protein (RhaB: 956 amino acids) with a MW of 106,049. Gene *rhaA* was showed to be homologous with *ramA* (41%) of α-L-rhamnosidase of *Clostridium stercorarium* (Zverlov *et al.*, 2000), while *rhaB* showed slight homology with enzymes from other sources.

On the comparative sequence analyses of lactic acid bacteria, the cloned α-L-rhamnosidase genes from different sources of LAB contain various amounts of amino acid residues. The cloned BdRham nucleotide from *Bifidobacterium dentium* consisted of a 2,673 bp sequence encoding a protein containing 890 amino acid residues. The cloned *BdRham* gene and amino acid sequence shared a very weak homology with the α-L-rhamnosidase genes and the corresponding proteins from *C. stercorarium*, *L. acidophilus*, and *L. plantarum*. The amino acid sequence of the cloned *BdRham* gene shared only 32%, 32% and 20% sequence identity with that of the α-L-rhamnosidases of *C. stercorarium*, *L. acidophilus*, and *L. plantarum*, respectively (Bang *et al.*, 2015). While a full-length gene encoding the α-L-rhamnosidase of *B. breve* ATCC 15700 encoded a 775-amino acid protein. And it had the same sequence as that reported in GenBank (Zhang *et*

*al.*, 2015). In the study of Michlmayr *et al.* (2015), two putative rhamnosidase genes (*ram*, *ram2*) of *P. acidilactici* DMS 20284 were expressed in *E. coli* with deduced protein sequences containing 653 and 525 amino acid residues, respectively. It was found that they had the highest sequence similarities with already characterized GH family 78 rhamnosidases $Ram1_{Lp}$ and $Ram2_{Lp}$ of *Lactobacillus plantarum* (Beekwilder *et al.*, 2009). Ram shared 56% identity with $Ram1_{Lp}$, Ram2 shared 61% with $Ram2_{Lp}$. Ram and Ram2 share 29 sequence identity with each other. While in the genome of *Lactobacillus acidophilus*, one α-L-rhamnosidase gene was found (*ramA*$_{La}$) displaying high similarity to that of α-L-rhamnosidases of *Clostridium stercorarium* (Zverlov *et al.*, 2000). The deduced amino acid sequences of the two α-L-rhamnosidases *rhaB1* and *rhaB2* from *L. plantarum* NCC245 share 26% identity with each other (Avila *et al.*, 2009). Among experimentally characterized α-L-rhamnosidases, RhaB1 shares 23 % identity with RhaB of *Bacillus* sp. GL1 and 22% with RhaB of *Thermomicrobia* PRI-1686, whereas RhaB2 shares 23% identity with RhaB of *Bacillus* sp. GL1 (Avila *et al.*, 2009).

In almost studies, after the α-L-rhamnosidase genes have been expressed in *E. coli*, the recombinant proteins were purified and the molecular mass as well as biochemical properties were determined. The molecular masses of α-L-rhamnosidases from different LAB were also different. The molecular masses of α-L-rhamnosidases from *L. plantarum* NCC245 were 73 kDa for RhaB1 and 57 kDa for RhaB2 (Avila *et al.*, 2009). Using size exclusion chromatography, molecular masses of Ram and Ram2 from *Pediococcus acidilactici* were 74 kDa and 241 kDa, respectively. The calculated molecular masses of Ram (76.8 kDa) and Ram2 (61.3 kDa) suggested that Ram would be a monomer and Ram2 a tetramer (Michlmayr *et al.*, 2011). The molecular mass of the α-L-rhamnosidase from *B. breve* (Zhang *et al.*, 2015) and from *B. dentium* (Bang *et al.*, 2015) determined by SDS-PAGE were approximately 87 kDa and 100 kDa, these are similar to the molecular masses reported for the extracellular α-L-rhamnosidases from fungi, including *A. aculeatus* (87 kDa) (Manzanares *et al.*, 2003), *A. niger* (85 kDa) (Manzanares *et al.*, 1997), *A. kawachii* (90 kDa) (Koseki *et al.*, 2008), and *A. nidulans* (102 kDa) (Manzanares *et al.*, 2000).

To assess the specificities of the α-L-rhamnosidases from different LAB sources, some potential substrates were used to test the ability to degrade rhamnosylated flavonoids such as rutin, nicotiflorin, narirutin, naringin and hesperidin. While $Ram1_{Lp}$ and $Ram2_{Lp}$ enzymes from *L. plantarum* efficiently converted the rutinosides rutin, nicotiflorin, narirutin, and hardly hydrolyzed naringin into prunin; whereas $RamA_{La}$ from *L. acidophilus* could convert all rutin, nicotiflorin, naringin and the majority of narirutin (Beekwilder *et al.*, 2009). α-L-rhamnosidases from *B. breve* and from *B. dentium* were able to hydrolyze both α-1,2 and α-1,6 glucoside linkage. They were thus able to hydrolyze rutin, hesperidin and naringin. However, both enzymes were more active

towards rutin (Bang *et al.*, 2015; Zhang *et al.*, 2015). It is so interesting that both enzyme Ram and Ram2 from *P. acidilactici* were unable to hydrolyze the natural flavanone glycoside naringin (Michlmayr *et al.*, 2011).

There are only few studies on the crystal structure of GH78 α-L-rhamnosidases (Rha78s) until now. The largest structure of Rha78s from *Streptomyces avermitilis* is SaRha78a composed of a single polypeptide chain of 1043 amino acids, which contains six distinct domains, including one α-domain and five β-domains (Fujimoto *et al.*, 2013). RhaB from *Bacillus* sp. GL1 is composed of five distinct domains. The rhamnosidase forms a homodimer in the crystal structure containing 1,908 amino acids, 43 glycerol molecules, four calcium ions and 1,755 water molecules. The RhaB structure consists of five domains, four of which are β-sandwich structures designated as domains N, D1, D2 and C, and an $(\alpha/\alpha)_6$-barrel structure designated as domain A (Cui *et al.*, 2007). However, KoRha from *Klebsiella oxytoca* consists of two domains only. Domain A, the catalytic domain, is mainly α-helical, consisting of residues 11–30 and 180–523, and contains the bound rhamnose. Domain B, the dimerization domain, is a β-sandwich domain consisting of residues 31–179 (O'Neill *et al.*, 2015). The catalytic domain of Rha78s is a typical $(\alpha/\alpha)_6$-barrel structure, and Rha78s hydrolyzes the glycoside bonds through general acid base assisted inverting mechanism (single displacement) (Cui *et al.*, 2007; Fujimoto *et al.*, 2013).

### 2.3.4. Assay of naringinase activity

There are several methods of following the enzymatic hydrolysis of naringin. Measuring the glucose and rhamnose increase by spectrophotometric methods and high-performance liquid chromatography (HPLC) is applied for evaluation of naringinase activity. Monitoring the naringin concentration and calculating the naringin decrease is complicated by the presence of prunin and naringenin. These procedures are presently available and used in many researches.

The earliest method for the determination of naringin and other flavanones was described by Davis (Davis, 1947). In this colorimetric method, alkaline diethylene glycol was used for measuring the content of flavones that may be present in citrus fruits, as well as in grapefruit in particular. From that the naringinase activity was calculated. The principle of the method based on the formation of a yellow chalcone produced by the reaction between naringin and diethylene glycol in alkaline solution (4N NaOH). The absorbance of product was measured at the wavelength of 420 nm. Because of its straightforward way, this method is used to assay the activity of naringinase.

Habelt *et al.* (1983) pointed out the major drawback of Davis method. There are difficulties in distinguish between the content of naringin, prunin and naringenin present in the reaction

mixture, because all three components have a common absorbance maximum at 420-430 nm. So, they gave a simple method for distinguishing between naringin, prunin and naringenin. The principle of the method was described as follows. Solution of naringin, prunin and naringenin gives stable yellow color when they react with strongly alkaline NaOH to form phenolate ions. Spectrophotometric measurement gives two maxima in absorbance: one for naringenin at 310 nm and one for both naringin and prunin, which have the same absorbance under these conditions, at 375 nm. Therefore, the quantity of naringenin is determined possible. In the second step, the treatment of the liberated aldohexoses with *o*-aminodiphenyl was applied to determine the sum of rhamnose and glucose. Then, thin-layer chromatography was used to quantify naringin, prunin and naringenin.

As mentioned above, naringinase is a complex enzyme containing α-L-rhamnosidase and β-D-glucosidase. In order to determine the naringinase activity, specific substrates are used to distinguish between these two enzymatic activities. For this purpose, p-nitrophenyl-α-rhamnopyranoside and p-nitrophenyl β-D-glucopyranoside can be specific substrates for α-L-rhamnosidase and β-D-glucosidase, respectively (Hashimoto *et al.*, 2003; Thammawat *et al.*, 2008; Avila *et al.*, 2009; Zhu *et al.*, 2017a)

In a study of Dunlap *et al.* (1962), a fungal enzyme preparation, "Naringinase C100", was separated by paper electrophoresis into individual rhamnosidase and glucosidase fractions. The specificity of these fractions in hydrolyzing a number of flavonoid and phenolic glycosides such as iso-quercitrin, pruning, esculin, scopolin, rutin, naringin, hesperidin, etc. was tested (Dunlap *et al.*, 1962). In another experiment, these authors developed paper chromatographic-fluorometric method for quantitative determinations of naringin and prunin in mixtures containing very small quantities of naringin, prunin and naringenin.

The synthetic substrate, *p*-nitrophenyl-α-L-rhamnosidase was used for assaying the α-L-rhamnosidase activity of naringinase photometrically, following the appearance of *p*-nitrophenolate anion (Romero *et al.*, 1985). The pH, temperature, or ionic strength optima of the enzyme did not change when using of this synthetic substrate. This is a specific method for measuring the α-L-rhamnosidase activity of naringinase, while the others are not. Compared to the HPLC method, it is quicker and also cheaper (20-fold greater).

A sensitive colorimetric method for naringin estimation was developed by using 2,2'-azino-bis-(3-ethylenzthiazoline-6-sulfonic acid) (ABTS) as peroxidase substrate. Under peroxidase activity, there is a coupling reaction of an ABTS radical cation with an oxidation product of naringin. So far, a purple-colored compound with a maximum absorbance at 560 nm is

formed (Arnao *et al.*, 1990). Arnao *et al.* (1990) also applied this method to assay the naringin content in grapefruit tissues.

Naringinase activity can be measured through the liberation of rhamnose and glucose. The 2,4-dinitrosalicylic acid (DNS) method (Miller, 1959) or Somogyi method (Somogyi, 1945) can be used to evaluate the reducing sugar (glucose). The determination of reducing sugar method (DNS method) also was considered as a reference method for assaying naringinase activity by Sigma. The DNS microassay procedure using a 96-microtiter plate was applied to analyze reducing sugars in the study on immobilized naringinase done by Vila-Real *et al.* (2010).

Ribeiro *et al.* (2008) developed and validated a HPLC method to control the debittering process of citrus juices using naringinase. This is a fast, effective method for the simultaneous determination and control of naringin and naringenin in grapefruit and oranges juices. The method is linear, precise, and selective for naringin and naringenin identification and quantification in citrus juices (Ribeiro & Ribeiro, 2008).

### 2.3.5. Characterization of naringinase

Naringinase from different sources has been characterized in some detail. The characteristic of naringinase and α-L-rhamnosidase as well as β-D-glucosidase expressed by naringinase were reported in many researches. Characteristics of naringinases from different sources are summarized in **Table 2.3**.

**Table 2.3 Characterization of naringinase from various sources**

| Enzyme | Source | pH | Temperature (°C) | Mol wt. (kDa) | pI | Stimulator | Inhibitor | $K_m$ (mM) | $V_m$ (U/mg) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| α-L-rhamnosidase | *Bacteriodes* JY-6 | 7.0 | | 240 | 4.2 | | L-rhamnose L-fucose, $Pb^{2+}$ | | | (Jang & Kim, 1996) |
| α-L-rhamnosidase | *Bacillus* sp. GL1 | 7.0 | 50 | 100 | | | $Cu^{2+}$, $Fe^{2+}$, $Hg^{2+}$, L-rhamnose | | | (Hashimoto *et al.*, 1999) |
| α-L-rhamnosidase | *Pseudomonas paucimobilis* FP2001 | 7.8 | 45 | 112 | 7.1 | $Ca^{2+}$ | | | | (Miake *et al.*, 2000) |
| Naringinase | *P. decumbens* PTCC 5248 | 4.5 | 55 | | | | Citric acid, glucose, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$ | 1.7 | | (Norouzian *et al.*, 2000) |
| α-L-rhamnosidase | *Pichia angusta* X349 | 6.0 | 40 | 90 | 4.9 | - | $Cu^{2+}$, $Hg^{2+}$ | | | (Yanai & Sato, 2000) |
| α-L-rhamnosidase | *A. aculeatus* | 4.5-5 | - | 85-92 | | | | | | (Manzanares *et al.*, 2001) |
| α-L-rhamnosidase | *A. terreus* | 4.0 | 44 | 96 | 4.6 | $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Co^{2+}$ | L-rhamnose | 0.17 | 84 | (Gallego *et al.*, 2001) |
| Naringinase | *A. niger* 1344 | 4.0 | 50 | 168 | | $Ca^{2+}$, $Co^{2+}$, $Mg^{2+}$ | $Hg^{2+}$, SDS, $Cu^{+2}$, $Mn^{2+}$ | | | (Puri & Kalra, 2005) |
| α-L-rhamnosidase | *A. kawachii* | 4.0 | 50 | 90 | | | | | | (Koseki *et al.*, 2008) |

| Enzyme | Source | pH | Temperature (°C) | Mol wt. (kDa) | pI | Stimulator | Inhibitor | $K_m$ (mM) | $V_m$ (U/mg) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| α-L-rhamnosidase | *P. ulaiense* | 5.0 | 60 | | | $Co^{2+}$ | $Hg^{2+}$ | 11 | 26 | (Rajal *et al.*, 2009) |
| α-L-rhamnosidase | *L. plantarum* NCC245 | 7.0<br>5.0 | 50<br>60 | 73<br>57 | | $Ca^{2+}$, $Co^{2+}$ | $Mn^{2+}$, $Fe^{2+}$, $Cu^{2+}$ | | | (Avila *et al.*, 2009) |
| α-L-rhamnosidase | *Pediococcus acidilactici* DSM 20184 | 5.5<br>4.5 | 50<br>70 | 74<br>241 | | | | | | (Michlmayr *et al.*, 2011) |
| Naringinase | *A. niger* | 4.5-5 | 45-55 | 131 | | | | | | (Ni *et al.*, 2012a) |
| α-L-rhamnosidase | *A. niger* | 4.5-5 | 50-60 | 87 | | $K^{+}$, $Ba^{2+}$ | $Fe^{2+}$, $Fe^{3+}$, $Zn^{2+}$, $Al^{3+}$, $Mn^{2+}$, $Cu^{2+}$, $Ag^{+}$, $Hg^{2+}$ | | | (Ni *et al.*, 2012b) |
| Naringinase | *A. aculeatus JMUdb058* | 4.0 | 50 | 69-348 | | | | 0.11 | | (Chen *et al.*, 2013) |
| α-L-rhamnosidase | *P. corylopholum* MTCC-2011 | 6.5 | 57 | 67 | | | | | | (Yadav *et al.*, 2013) |
| Naringinase | *A. brasiliensis* MTCC1344 | 6.0 | 60 | - | - | - | $Hg^{2+}$, EDTA, SDS | 3.21 | 321 | (Shanmugaprakash *et al.*, 2015) |
| α-L-rhamnosidase | *B. breve ATCC 15700* | 5.5-7 | 55 | 87 | - | | | 2.2 | 56.4 | (Zhang *et al.*, 2015) |
| α-L-rhamnosidase | *B. dentitum* | 6.0 | 35 | | | $Ca^{2+}$, $Mg^{2+}$ | $Cu^{2+}$ | | | (Bang *et al.*, 2015) |
| Naringinase | *A. oryzae* 11250 | 5.0 | 45 | | | | $Ag^{2+}$ | 1.6 | | (Zhu *et al.*, 2017a) |
| Naringinase | *A. oryzae* 11250 | 5.0 | 45 | 23 | | | $Li^{+}$, $Mn^{2+}$, $Pb^{2+}$, $Ba^{2+}$, | 1.6 | | (Zhu *et al.*, 2017a) |

| Enzyme | Source | pH | Temperature (°C) | Mol wt. (kDa) | pI | Stimulator | Inhibitor | $K_m$ (mM) | $V_m$ (U/mg) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $Al^{3+}$, $Cu^{2+}$, EDTA | | | |
| Naringinase | *Bacillus amyloliquefaciens* 11568 | 7.5 | 45 | 32 | | | $Zn^{2+}$, $Ag^{+}$, $Ba^{2+}$, $Fe^{2+}$, $Hg^{2+}$, $Cu^{2+}$ | | | (Zhu *et al.*, 2017b) |
| Naringinase | *Cryptococcus albidus* | 5.0 | 60 | 50 | | | $Ag^{+}$ | | | (Borzova *et al.*, 2018) |
| α-L-rhamnosidase | *Bacteriods thetaiotaomicron* VIB-5482 | 6.5 | 60 | 86 | | | | | | (Li *et al.*, 2018) |

### 2.3.6. Application of naringinase

Naringin and its hydrolyzed products have a potential application in pharmaceutical and food industries. So naringinase has been used mainly in hydrolyzing naringin to debitter citrus juices and transform steroids.

#### 2.3.6.1. Debittering of fruit juices

Bitterness in citrus fruits is primarily related to two compounds – naringin and limonin. Naringin (4',5,7-trihydroxyflavanone-7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside) is known as to be the main bitter component in grapefruit and affects acceptance by consumers. In order to debitter, some techniques have been reported in previous studies. β-cyclodextrin was used in reduction of limonin and naringin (Konno *et al.*, 1981; Mongkolkul *et al.*, 2006). Adsorption technique have been studied to lower the content of bitterness in citrus juice (Jungsakulrujirek & Noomhorm, 2004), but this method has some drawbacks affecting juice acidity, flavor, sweetness and turbidity as well as it is less efficient (Ribeiro & Ribeiro, 2008). The application of enzymatic hydrolysis in reduction of naringin concentration is a promising technique, because this method can control the quality and improve the commercial value of citrus juices beside maintaining health properties and increasing acceptance by the consumers (Ribeiro & Ribeiro, 2008). Lead by the goal to reduce the bitterness in citrus fruits, many achievements have been reported on different products such as orange juices, white and red grapefruit juices and kinnow, etc. using enzymatic hydrolysis (Puri & Banerjee, 2000; Prakash *et al.*, 2002; Şekeroğlu *et al.*, 2006; Ferreira *et al.*, 2008; Ribeiro *et al.*, 2008; Ni *et al.*, 2012a; Ni *et al.*, 2014; Zhu *et al.*, 2017a; Zhu *et al.*, 2017b). Naringinase has been used as purified enzyme extracted from bacterial sources. Immobilization also was applied to reduce the bitter taste of juice.

One of the early studies on debittering citrus juice by naringinase was done by Olson *et al.* (1979). Commercial naringinase from *Aspergillus niger* was immobilized in a hollow fiber reactor to hydrolyze naringin in grapefruit juice by pumping unclarified juice though the reactor. A high correlation was achieved between sensory perception of bitterness in grapefruit juice and lowered naringin levels in the juice produced by the hollow fiber/naringinase reactor (Olson *et al.*, 1979).

Application of naringinase is not restricted to citrus fruits but works also in other fruits such as palmyrah fruit. The main bitter component of palmyrah fruit pulp is identified as a tetra glycoside of spirost-5 en-3β-ol, named as flabelliferin II, containing two glucose and two rhamnose residues. Bitterness can be removed by naringinase action to release rhamnose and glucose forming other flabelliferins. A beverage with a pleasant mango cordial-like color, flavor and texture was produce by application of naringinase (Jansz *et al.*, 1994).

Naringinase encapsulated sodium alginate beads were investigated for reduction of the bitter taste of kinnow juice (Puri *et al.*, 1996a). An optimal matrix of 2% sodium alginate for naringinase immobilization demonstrated a 60% reduction of bitterness. Different methods using naringinase encapsulated in calcium alginate beads to debitter juices were presented by Ferreira *et al.* (2005), Pedro *et al.* (2007) and Ribeiro *et al.* (2010) with high-pressure conditions. At 160 MPa, naringinase entrapped in Ca-alginate beads displayed higher activity, as well as 65% higher maximum initial rate, and 70% lower $K_M^{ap}$ as compared to treatment at atmospheric pressure (Pedro *et al.*, 2007). Effect of different levels of high pressure combined with naringinase immobilized in calcium alginate beads on naringin hydrolysis was evaluated. While at 160 MPa and 37 °C a 50% increase in the concentration of reducing sugars was obtained compared to the reaction at atmospheric pressure; under high pressure of 200 MPa, the naringenin concentration of 33 mg/L was obtained at 54 °C, which corresponds to a naringin reduction of 72% at 160 MPa. At atmospheric pressure (0.1 MPa), the naringin reduction was only 35% (Ferreira *et al.*, 2008). The authors concluded that debittering of about of 75% can be achieved under the pressure of 160 MPa at 37 °C for 20 min with naringin hydrolysis by naringinase immobilized in calcium alginate beads.

Response surface methodology was used to model the enzymatic hydrolysis by naringinase to remove the bitter taste from juice. A 81% naringin conversion was achieved at 60 °C and 205 MPa after 30 min of reaction (Ribeiro *et al.*, 2010). High pressure as a none thermal preservation technology is often used as an alternative or complementary process to heat treatment. So hydrolysis of naringin under high pressure could also sterilize citrus juice as well as preserve the volatile compounds, vitamins, pigments and other compounds associated with sensory qualities.

Immobilization of naringinase on different carriers was also investigated. For example, naringinase from *Penicillium* sp. was immobilized in cellulose acetate films (Soares & Hotchkiss, 1998b). Immobilization of naringinase on glutaraldehyde coated hen egg white through 1% glutaraldehyde cross linking was presented (Puri *et al.*, 2001). Preparation of polyvinyl alcohol with the crosslinking agent glutaraldehyde to immobilize naringinase was able to reduce the naringin content of grapefruit juice during storage (Nobile *et al.*, 2003). Naringinase produced by *Aspergillus niger* CECT 2088 was immobilized into a polymeric matrix consisting of polyvinyl alcohol (PVA) hydrogel (Busto *et al.*, 2007). In another study, PVA–alginate beads developed with thermal, mechanical and chemical stability to high temperatures (<80 °C) was used to immobilized naringinase from *Penicillium decumbens* (Nunes *et al.*, 2010). Mesoporous silica MCM-41 was used to bound naringinase via adsorption with glutaraldehyde and used to debitter white grapefruit (Lei *et al.*, 2011).

Recently, study on reducing bitterness in citrus juice has been continuously developed. Determination of the effectiveness of different naringinase concentration on reducing naringin in "Biblia sweet" oranges to enhance its quality was presented. The optimum reduction (86%) could be obtained by adding 1.0g/L naringinase enzyme and incubating at 50 °C for 4 hours (De Silva *et al.*, 2017). Application of purified naringinase from *Bacillus amyloliquefaciens* 1168 (Zhu *et al.*, 2017b) and from *Aspergillus oryzae* 11250 (Zhu *et al.*, 2017a) also provided promising results in debitterization of citrus juice.

Debittering of citrus juice accomplished via hydrolysis has great potential in improvement of quality and commercial value. This process also increases the acceptance of the product by the consumer due to maintaining health properties of citrus juice. In the study of Ribeiro *et al.* (2008), the reduction of naringin was achieved by hydrolysis with naringinase, and the anti-inflammatory potentials of naringin and naringenin also were evaluated. A 95% naringin conversion in grapefruit juice was obtained with immobilized naringinase in *K*-carrageenan. More importantly, interesting results were obtained regarding anti-inflammatory properties of naringin and naringenin in the juice. Rats were administered orally grapefruit juice (before and after processing), and it was found that enzymatic processing did not affect the anti-inflammatory properties of the juice, a reduction of approximately 90% on paw edema after 6 h of administration was observed (Ribeiro *et al.*, 2008).

### 2.3.6.2. Enhancement of wine aroma

The commercial glycosidase Cytolase PCL5 from *Aspergillus niger*, containing β-glucosidase, α-arabinosidase, and α-rhamnosidase, which were considered suitable for aroma enhancement in wine making, was immobilized to a solid carrier (Caldini *et al.*, 1994). The aim of this study was to develop a continuous process for wine aroma enhancement. In the study of Orejas *et al.* (1999), it was shown that α-L-rhamnosidase from *Aspergillus nidulans* was only slightly affected by glucose and $SO_2$, and partly inhibited by ethanol. These results proved that this enzyme has a potential in wine aroma release (Orejas *et al.*, 1999a). Purified α-L-rhamnosidase from *Aspergillus niger* was applied to increase aroma of a model wine solution containing aromatic precursors extracted from the skins of Moscato grapes. An increase in the content of total terpenols of ca. 2.3 times was observed (Spagna *et al.*, 2000). The study of factors affecting the activity of α-L-rhamnosidase from *Aspergillus tereus* in winemaking showed that ethanol (12% v/v) and glucose (21% w/v) decreased enzyme activity by approximately 20%, while this was not affected by $SO_2$ (Gallego *et al.*, 2001).

The abilities of α-rhamnosidase from *Pediococcus acidilactici* to release grape derived terpenes in combination with β-glucosidase and naringinase were determined with a modified Muscat wine (Michlmayr *et al.*, 2011). Significant increase in concentration of terpenes (monoterpenes linalool and cis-linalool oxide) could be observed in all cases. α-rhamnosidase of Patagonian indigenous yeast was shown to be able to increase the content of aromatic compounds in grape juice (Rodriguez *et al.*, 2010).

Application of naringinase in production of rhamnose as well as prunin was considered (Vila-Real *et al.*, 2010). Many drugs and precursors were prepared by α-L-rhamnosidase by biotransformation of steroids (Feng *et al.*, 2007). Production of ginsenosides (Ko *et al.*, 2003) or glycolipids (Saerens *et al.*, 2009) were achieved with naringinase.

## 2.4. Probiotic microorganism

### 2.4.1. Introduction

Probiotics are defined as live microbial food supplements which benefit the health of consumers by maintaining or improving their intestinal microbial balance (Fuller, 1989). Alternatively, probiotics are defined as "live microorganisms, which, when administered in adequate amount, confer health benefits on the host" (FAO/WHO, 2002).

The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium*. Both lactobacilli and bifidobacteria are Gram-positive lactic acid-producing bacteria that constitute a major part of the normal intestinal microflora in animals and humans.

Lactobacilli are non-spore forming rod-shaped bacteria. They have complex nutritional requirements and are strictly fermentative, aerotolerant or anaerobic, acidic or acidophilic. Lactobacilli are found in a variety of habitats where rich, carbohydrate-containing substrates are available, such as human and animal mucosal membranes, on plants or material of plant origin, sewage, fermented milk products, and fermenting or spoiling food (De Vrese & Schrezenmeir, 2008).

Bifidobacteria constitute a major part of the normal intestinal microflora in humans throughout life. They appear in the stools a few days after birth and increase in number thereafter. The number of bifidobacteria in the colon of infant is $10^{10}$–$10^{11}$ cfu/gram, but this number decreases with age (De Vrese & Schrezenmeir, 2008). Bifidobacteria are nonmotile, nonsporulating, rods with varying appearance. Most strains are strictly anaerobic.

While conventional starter cultures, above all, have been optimized in respect to technological and sensory properties as well as culture stability in acidified milk, probiotic microorganism strains have been selected from the broad spectrum of lactic acid bacteria and other

microorganisms for their health-promoting qualities (De Vrese & Schrezenmeir, 2008; Nagpal *et al.*, 2012b).

### 2.4.2. Benefits of probiotic

Probiotics are used to improve the health of both animals and humans through the modulation of the intestinal microbiota. Several well-characterized strains of lactobacilli and bifidobacteria are available for human use to reduce the risk of gastrointestinal infections or treat such infections. A huge number of studies on beneficial effects of probiotic consumption were investigated including improvement of intestinal health by the regulation of microbiota, stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, and reducing the risk of certain other diseases (Salminen *et al.*, 2005; Parvez *et al.*, 2006; Yadav *et al.*, 2008; Kumar *et al.*, 2009; Nagpal *et al.*, 2010; Kumar *et al.*, 2011; Nagpal & Kaur, 2011; Nagpal *et al.*, 2012a).

### 2.4.3. Properties essential for effective and successful probiotics

For the purpose of health-promoting qualities, a number of selection criteria were established.

- Safe for humans, i.e. free of pathogenic and toxic effects, and best adapted to the ecosystem of the gut.
- Tolerance to gastric and bile acid as well as sufficient resistance against digestive enzymes enable the survival during the passage through stomach and upper intestinal tract, and have health-promoting effects in the gut.
- Detection of parameters enabling a (positive) influence on the intestinal flora like adhesion to intestinal epithelial cells, survival and reproducing capacity in the human large intestine, or production of antimicrobial substances. A permanent colonization of bacteria in the large bowel has not been proven. It is not requested for attaining probiotic qualification, as far as a daily or at least regular bacteria supply occurs via regular intake of probiotics.
- Probiotic bacteria must also comply with the technological requirements, and a certain probiotic content must be guaranteed until the expiry of shelf life.

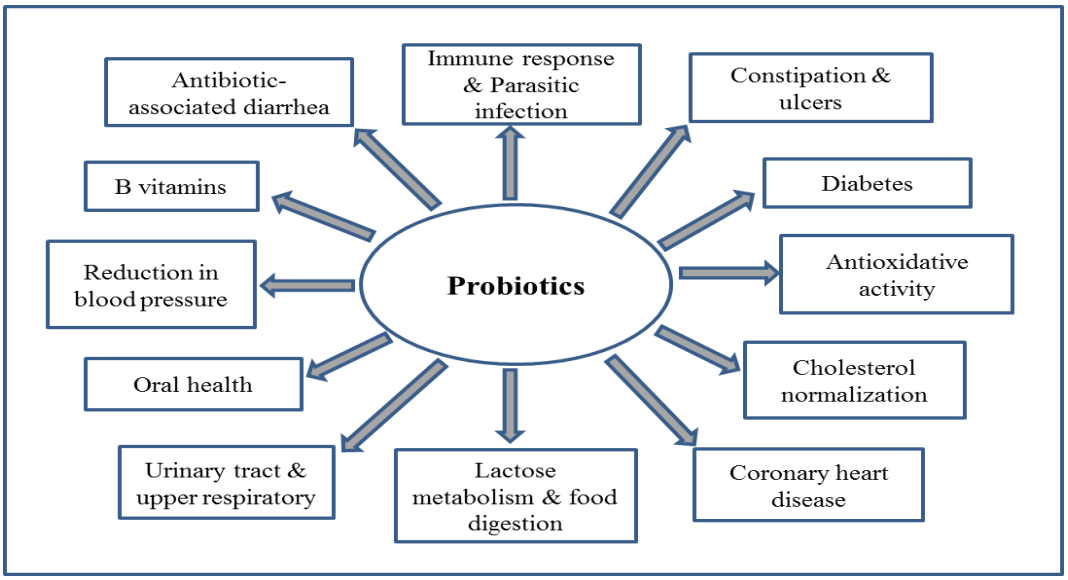


**Figure 2.6 Health benefits attributed to probiotics**
(Kechagia *et al.*, 2001; Nagpal *et al.*, 2012b)

## 2.5. Probiotic beverage

In recent years, the awareness of the consumers leads to the food related to the health called "healthy foods". This phenomenon could be partly attributed to the increasing cost of healthcare, the steady increase in life expectancy, and the desire of older people for an improved quality of their later years (Luckow *et al.*, 2006). The healthy foods were generally labeled "functional food", which food exerts beneficial effects or more specific body functions, in addition to the traditional nutritional effects. Well-known examples of functional foods are those containing or prepared with bioactive compounds, such as dietary fiber, oligosaccharides, and active "friendly" bacteria that promote the equilibrium of useful intestinal bacterial strains (Jankovic *et al.*, 2010; Perricone *et al.*, 2015). One of the combinations is "functional beverage", when probiotics are introduced to the fruit juice. Fruit juice is positioned as a healthy food product and is currently consumed frequently and loyally by a large percentage of the global consumer population. Furthermore, juices do not contain any dairy allergens (lactose, some peptides etc.) that might prevent usage by certain segments of the population (Luckow & Delahunty, 2004). Recently, beverages based on fruits, vegetables, cereals and soybeans have been proposed as new products containing probiotic strains; particularly, fruit juices have been reported as a novel and appropriate medium for probiotics for their content of essential nutrients. Many reports focused on the health benefits of fruit juice. Therefore, the combination of fruit juice with probiotic bacteria will create a beverage with potential health benefits.

Fruit juices are rich sources of vitamins, minerals (electrolytes), dietary fibers, carbohydrates, and several other phytochemicals like flavonoids and carotenoids. So, it is suitable

to choose fruit juices as probiotic carriers. Many authors reported on the lactic fermentation of fruit juice by probiotic strains from different kind of fruits such as orange, apple, pineapple, noni, apricot, fig and pomegranate (Tien *et al.*, 2005; Wang *et al.*, 2009; Mousavi *et al.*, 2010; Marhamatizadeh *et al.*, 2012; Bevilacqua *et al.*, 2013; Costa *et al.*, 2013; Khezri *et al.*, 2016; Bujna *et al.*, 2018). This reveals that probiotic bacteria can grow well in fruit juice. In some cases, application of lactic fermentation by probiotic bacteria even increased the antioxidant capacity of fruit juice (Bujna *et al.*, 2018).

## 2.6. Factors affecting lactic fermentation

### 2.6.1. Nutritional requirements

Carbon can be present in the culture medium in the form of sugars, amino acids and organic acids that have high energy content (Cui *et al.*, 2011). Nitrogen can be supplied to the culture media as peptone, yeast extract, urea or ammonium sulfate. These nitrogen sources, available in the form of amino acids, peptides and inorganic compounds, can be implied either in anabolic or catabolic processes (Nancib *et al.*, 2001). Mineral elements (Mg, Mn and Fe), which are provided in the medium in the form of salts ($MgSO_4$, $MnSO_4$ and $FeSO_4$) (Gupta & Gandhi, 1995; Fitzpatrick & O'keeffe, 2001), and vitamins (mainly belonging to the B group) present in yeast extract are essential elements that act as cofactors in many enzymatic reactions.

Some reports proved that metabolism of carbohydrates by *Lactobacillus* spp. depends on the strain, the substrate and even on the fermentation time (Hou *et al.*, 2000). Glucose is a very good carbon and energy source for lactobacilli and bifidobacteria (Hou *et al.*, 2000).

### 2.6.2. pH

Lactic acid bacteria (LAB) grow preferentially at pH between 5.0 and 7.0, the medium acidification associated with lactic acid production inhibits fermentation (Nomura *et al.*, 1987; Roberto *et al.*, 2007). The use of ammonium hydroxide for pH control instead of sodium hydroxide enhances the final concentration as well as the rate of product biosynthesis. The ammonium hydroxide is used not only for pH control but also as a nitrogen source for LAB (Mercier *et al.*, 1992). For production of fermented food, acid tolerance is an important characteristic of probiotics for surviving during fermentation in food medium (Holzapfel & Schillinger, 2002). Low pH of medium can lead to the decrease in the maximum growth rate and an extended length of the lag phase (Yáñez *et al.*, 2008). For lactic acid fermentation of fruit juice, pH is one of the most important factors affecting the survival of probiotics. Juices contain a high level of organic acids and have low pH values. Lactobacilli are generally resistant, and survive in juices with pH ranging

from 3.7 to 4.3; on the other hand, bifidobacteria are less acid tolerant, and a pH of about 4.6 is detrimental for their survival (Tripathi & Giri, 2014).

### 2.6.3. Temperature

The temperature has significant effect on the fermentation time as well as on the fermentation ability of probiotics in fermented products. The fermentation time depends on the optimum temperature of bacterial growth. Most lactic acid bacteria have optimum growth temperature between 20 and 45°C (Martinez *et al.*, 2013), so the optimal growth conditions vary depending on the producers. Some studies showed that the optimum temperatures for *Lactobacillus casei* and *L. casei* subsp. *rhamnosus* are 37 and 45°C (Hujanen & Linko, 1996),

### 2.6.4. Substrate inhibition

Substrate inhibition seems to depend on both the microorganism and the carbon source. An increase in the initial glucose concentration was shown in fact to delay the growth of *L. delbrueckii* and *L. bulgaricus*, reducing both the specific productivity and product yield (Burgos-Rubio *et al.*, 2000). Such an inhibition was not observed using *L. casei* on sucrose up to 100 g/L (Büyükkileci & Harsa, 2004), *L. brevis* and *L. pentosus* on xylose up to 20 g/L (Garde *et al.*, 2002), and *L. helveticus* on lactose up to 110 g/L (Schepers *et al.*, 2002). However, xylose inhibition of *L. lactis* fermentation was an order of magnitude stronger than that exerted by glucose (Ishizaki *et al.*, 1993).

### 2.6.5. Product inhibition

Lactic acid was shown to exert an inhibitory effect on cell growth, which is stronger than that on fermentation activity (Milcent & Carrere, 2001; Madzingaidzo *et al.*, 2002). Lactic acid inhibition on cell proliferation and metabolism is possibly due to the increase in medium osmotic pressure, and also some fermentation by-products such as formic acid, acetic acid or sodium formate may exert individual inhibitory effects (Loubiere *et al.*, 1997; Lin *et al.*, 2008).

# 3. MATERIALS AND METHODS

## 3.1. Chemicals

Naringin, diethylene glycol, tri[2-pyridyl]-s-triazine reagent, Folin-Ciocalteu reagent, and L-rhamnose were of analytical grade and purchase from Sigma–Aldrich (Hungary). All other chemicals for preparation of media (MRS, TPY, Beeren's) were purchased either from Reanal (Hungary) or VWR (Hungary).

## 3.2. Screening naringinase production of probiotic bacteria

Bacteria from the fermentation lab of our Research Centre, including 16 strains of *Lactobacillus* and 20 strains of *Bifidobacterium*, were screened for naringinase production.

*Lactobacillus* ssp. were cultivated in MRS broth (De Man *et al.*, 1960) containing (per liter) proteose peptone 10 g, yeast extract 8 g, meat extract 8 g, glucose 20 g, sodium acetate 5 g, ammonium citrate 2 g, $K_2HPO_4$ 2 g, $MgSO_4$ 0.2 g, $MnSO_4$ 0.05 g and 1 mL of Tween80.

*Bifidobacterium* spp. were grown in TPY broth (Trypticase-Phytone-Yeast extract) containing (per liter) trypticase peptone 10 g, Phytone peptone 5 g, glucose 5 g, yeast extract 2.5 g, Tween80 1 mL, cysteine-HCl 0.5g, $K_2PO_4$ 2 g, $MgCl_2.6H_2O$ 0.5 g, $ZnSO_4.H_2O$ 0.25 g, $CaCl_2$ 0.15 g, $FeCl_3$ 0.03 g.

For screening of naringinase activity, the *Lactobacillus* spp. were grown in MRS broth without meat extract and lower glucose concentration (3 g/L), supplemented with 0.05% naringin, incubated aerobically at 37 °C for 24 hours. Bifidobacteria were grown for production of naringinase in TPY broth modified with 0.05% naringin, and incubated anaerobically (in Bugbox anaerobic chamber, Ruskin Technology) at 37 °C for 24 hours.

The ability of naringinase production was checked for all strains, and the best naringinase producing bacterium was chosen for further investigation.

## 3.3. Effect of some factors on naringinase production by *L. fermentum* D13

### 3.3.1. Effect of inoculum ratio of bacteria

*L. fermentum* D13 was chosen to study naringinase production. To study the effect of inoculum ratio of bacteria on enzyme production, from the cell culture of $10^9$ cfu/mL, 2%, 4%, 6%, and 8% was added to the MRS medium that was supplemented with 0.05% naringin and the glucose content was adjusted to 3 g/L.

### 3.3.2. Effect of different pH

The effect of initial pH on growth and production of naringinase from *L. fermentum* D13 was checked in 100 mL Erlenmeyer flask containing 50 mL medium. The initial pH of the MRS fermentation medium ranged from 5.0 to 8.0 with steps of pH 1.0 unit. The pH was adjusted by 1N NaOH or 1N $H_2SO_4$ depending on the pH level needed. The optimum inoculum ratio of bacteria was applied to find the best pH for production of naringinase.

### 3.3.3. Effect of naringin concentration

Different naringin concentrations from 0.05% to 0.2% with the step 0.05% was added to the MRS medium to check the influence of inducer to naringinase activity under optimal pH and optimal ratio of bacteria inoculated to the fermentation medium.

### 3.3.4. Effect of carbohydrate sources

Nine different carbohydrate sources at the concentration of 1% (w/v) were used in MRS medium containing 0.05% naringin for studying suitable carbohydrate source for naringinase production. They were glucose, starch, rhamnose, lactose, maltose, sucrose, fructose, molasses and galactose.

### 3.3.5. Effect of metal ions

To study the effect of different metal ions on enzyme production, the salts of different metal ions including $Fe^{3+}$, $Ca^{2+}$, $Mg^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$ and $Ni^{2+}$ were added to the fermentation medium.

## 3.4. Optimization of medium components for naringinase production

The best conditions for naringinase production by *L. fermentum* D13 was established via Response Surface Methodology (RSM). In this study, the central composite design (CCD) was used to investigate the effects of three independent variables, chosen by the results of the preliminary experiments, for optimization of the medium components for naringinase production. The ranges of variables were concentration of sucrose (2.5 – 7.5 g/L), of naringin (0.05 – 0.15 %), and pH (5.5 – 6.5) each coded -1, 0, and +1 (**Table 4.3**). The central composite design consisting of 17 experimental points including three replications at the central point (0) was applied to find the optimal levels of each of the significant variables. The naringinase production, pH optimum, optimal naringin and sucrose contents were analyzed using the analysis of variance method (ANOVA) combined with the Fisher's test.

Both linear and quadratic effects of the three variables under study, as well as their interaction on naringinase production were calculated. Experimental data were fitted to a second-order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model used in the response surface analysis was as follows:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + {}_{23}X_2X_3$$

where $a_0$, $a_1$, $a_2$, $a_3$, $a_{11}$, $a_{22}$, $a_{33}$, $a_{12}$, $a_{13}$, $a_{23}$ are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and $X_1$, $X_2$, $X_3$ are independent variables pH, naringin and sucrose contents.

The fit of the models was evaluated by the determination coefficients ($R^2$) and adjusted $R^2$ ($R^2$-adj). Optimal conditions for naringinase production in model depended on pH, naringin and sucrose contents. Three-dimensional surface plots were drawn to the interaction effects of the independent variables on the dependent variable, being described by the polynomial equation, which was fitted to the equation data.

### 3.5. Characterization of crude enzyme naringinase

The culture broth after 24 hours of fermentation was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected for lyophilization at 0.25 MPa, 17 °C. The crude enzyme was used to investigate the effect of pH, temperature and different metal ions on naringinase activity.

#### 3.5.1. Effect of pH on the activity of crude naringinase

The effect of pH on the activity of naringinase was studied at 40 °C in eight different buffers (0.1 M) with a range of pH values (3.6–7.0): acetate buffer (pH 3.6–5.5), sodium phosphate buffer (pH 6.0–7.0). Enzyme activities were measured by the standard assay procedure.

#### 3.5.2. Effect of temperature on crude naringinase activity

The optimum temperature for naringinase activity was determined by incubating the enzyme at different temperatures (30–55 °C) in 0.1 M acetate buffer (pH 4.0). The enzyme activities were measured by standard assay procedures as described in 3.7.1.

#### 3.5.3. Effect of different metal ions on crude naringinase activity

The influence of metal ions on naringinase activity was investigated in 0.1 M acetate buffer of pH 4.0 at 40 °C. Crude naringinase was incubated in the presence of metal ions $Cu^{2+}$, $K^+$, $Mn^{2+}$,

$Zn^{2+}$, $Fe^{3+}$,$Al^{3+}$, $Ca^{2+}$ and $Mg^{2+}$ with concentrations of 5 mM, and its activity was compared with one of the control without metal ions.

## 3.6. Application of probiotic lactic bacteria for debittering of grapefruit juice

### 3.6.1. Grapefruit juice

Pasteurized grapefruit juices were purchased from the supermarket. The initial pH of the grapefruit juice was adjusted to pH 6.3 with 4N NaOH before fermentation.

### 3.6.2. Strains and cultures

Probiotic lactic acid bacteria (*Lactobacillus plantarum 01, Lactobacillus rhamnosus B01725, Lactobacillus fermentum D13, Bifidobacterium bifidum B7.5*) were from the fermentation lab of Research Centre for Bioengineering and Process Engineering, Faculty of Food Science, Szent István University, Hungary.

All strains of *Lactobacillus* were cultured in MRS medium at 37 °C for 24 hours. *B. bifidum B7.5* was cultivated in TPY medium at 37 °C for 24 hours under anaerobic conditions (in Bugbox anaerobic chamber, Ruskin Technology, USA).

### 3.6.3. Fermentation of grapefruit juice with probiotic lactic acid bacteria

Grapefruit juice (70 mL) was inoculated in 100 mL flask with *Lactobacillus* and/or *Bifidobacterium* strain and kept under aerobic conditions (in case of mono culture of *Lactobacillus*) or anaerobic condition by using Bugbox anaerobic chamber (in case of mixed cultures or *B. bifidum B7.5*) at 37 °C for 24 hours. Samples were taken during fermentation process every 6 h and every 2 weeks during the storage of 3 months at 4 °C. From the samples, the number of the colony-forming units (cfu) of bifidobacteria and lactobacilli were determined. In addition, the pH, antioxidant capacity, and total polyphenol content of fermented grapefruit juice also were measured. The carbohydrate concentrations, organic acid contents, and naringin content of the juices were determined during the 24 h of fermentation.

## 3.7. Analytical methods

### 3.7.1. Determination of naringinase activity

The culture broth after 24 hours of fermentation was centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was collected for estimation of naringinase activity (**Fig. 3.1**). The activity of naringinase was assayed based on the spectrophotometric method of Davis (Davis, 1947) with modification (Puri *et al.*, 2005). Briefly, the mixture containing 0.8 mL of 0.1% naringin dissolved in 0.1 M acetate buffer (pH 4.0) and 0.2 mL culture filtrate was incubated at 40 °C for 60 min. Then, 50 µL aliquot was added to 2.5 mL of 90% diethylene glycol followed by the addition of 50 µL 4N NaOH. The samples were stirred and kept at room temperature for 15 min. The substrate (naringin) and the hydrolyzed products (prunin, naringenin) react with reagent forming the yellow color mixtures, but the color intensity of these are different. The absorbance of yellow chalcones were measured at the wavelength of 420 nm. One unit of naringinase activity was defined as the amount of enzyme that could hydrolyze 1 µg of naringin per mL and minute at the assay conditions.

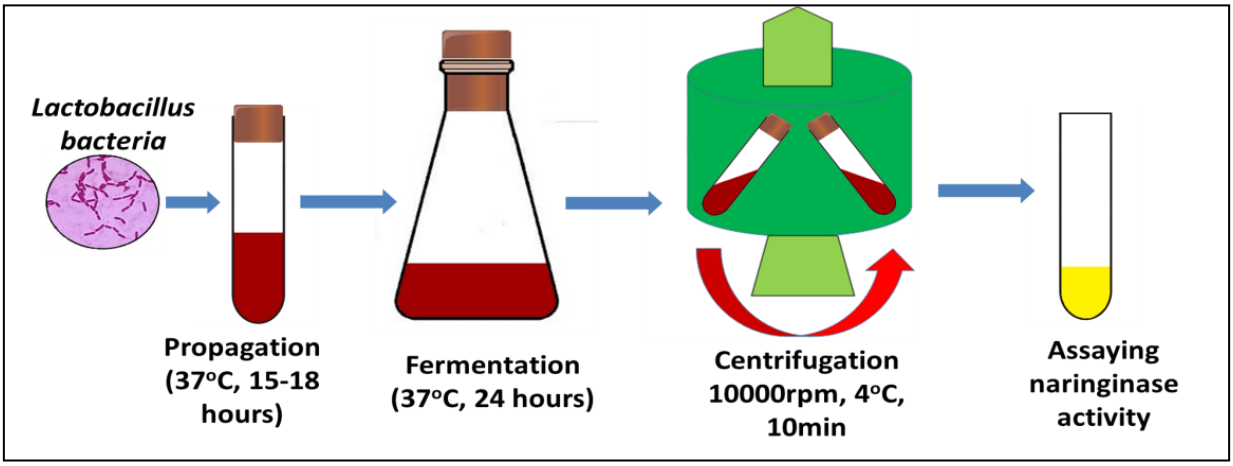


**Figure 3.1 Procedure of naringinase activity analysis**

### 3.7.2. Determination of biomass

A sample of culture broth (20 mL) was centrifuged at 12000 rpm for 10 min. Then the sediment cells were washed twice with deionized water followed by centrifugation. Biomass was calculated as of biomass (dry weight) produced per liter of fermentation broth (Puri *et al.*, 2010; Puri *et al.*, 2011a).

### 3.7.3. Determination of protein content

The protein content of samples was determined by the Bradford dye-binding procedure (Bradford, 1976) using Bio-Rad Protein Assay Kit (Bio-Rad, USA). Dye reagent was prepared by diluting 1 part of dye reagent concentrate with 4 parts of distilled, deionized water. An aliquot of sample (100µL) was added to 5 mL of diluted dye reagent and vortexed. The sample was incubated at room temperature for at least 5 min and absorbance was measured at 595 nm.

### 3.7.4. Enumeration of probiotic microorganisms

Samples from fermented grapefruit juices were diluted to $10^{-7}$–$10^{-5}$ by successive 10-fold dilution with sterile 0.85% sodium chloride solution. The aliquots of dilutions were transferred into Petri dishes and mixed with the appropriate medium. The MRS agar was used to determine the cell number of lactobacilli in grapefruit juices fermented by mixed cultures and monoculture. Beeren's agar medium was used to determine the cell number of bifidobacteria in grapefruit juices fermented by mixed cultures. TPY agar medium was used to determine the cell number of bifidobacteria in grapefruit juices fermented by mono culture. The incubation was done anaerobically in the case of bifidobacteria and aerobically in the case of lactic acid bacteria. The colonies were counted after 48-72 hours of incubation at 37 °C.

### 3.7.5. Analysis of carbohydrates and organic acids

The samples of fermented grapefruit juice were centrifuged at 14000 rpm for 10 min. Then the cell-free supernatant of grapefruit juices was used to determine the concentration of sugars and organic acids by HPLC methods. The Surveyor HPLC system (Thermal Scientific Corporation, USA) consisting of a quadruple pump, an autosampler, a refractive index detector (RI), a photodiode array (PDA) detector, and columns of Hi-Plex H or Hi-Plex Ca 7.7 x 300 mm (Agilent, USA) equipped with column oven was applied. 5 mM $H_2SO_4$ or dd. water was used as the mobile phase. Isochromatic elution mode with 0.6 mL/min flow rate was applied. Injection volume was 10 μL. The temperature of the column was maintained at 45 °C, and running time was 25 min. The data acquisition and integration were performed using the ChromQuest 5.0 software package. Standards of organic acids (lactic, acetic, oxalic, butyric, citric, succinic and malic acid) and sugar standards (glucose, fructose, sucrose) were applied to qualify and quantify the organic acids and sugars. All standard of organic acids and sugars were supplied by Sigma-Aldrich (Hungary).

### 3.7.6. Analysis of antioxidant capacity

The ferric-reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996) was used to measure the total antioxidant content of the fermented grapefruit juices. The FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue colored ferrous-tripyridyltriazine complex from colorless oxidized ferric form by the action of electron donating antioxidants. 10-fold dilutions of centrifuged samples of fermented grapefruit juice were made with deionized

water. An aliquot of the diluted sample (50 μL) was added to 1.5 mL of FRAP reagent then incubated at 37 °C for 10 min. The absorbance of the colored sample was measured spectrophotometric at 593 nm. The standard curve was obtained with different concentrations of $FeSO_4$ solution (mM). Unicam Helios UV/Vis spectrophotometer was used to measure the absorbance.

FRAP reagent was prepared by mixing 200 mL acetate buffer pH 3.6; 20 mL of 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) 10 mM solution, in which TPTZ was dissolved in 40 mM HCl solution; and 20 mL of ferric chloride 20 mM.

### 3.7.7. Determination of total polyphenol content

The total phenolic content (TCP) of the samples was determined by using the Folin-Ciocalteu colorimetric method with some mini modifications. 20-fold dilutions of centrifuged samples of fermented grapefruit juice were made with deionized water. An aliquot of diluted sample (200 μL) was added to 1 mL of Folin-Ciocalteu reagent (10%). After 8 min, 0.8 mL of Na2CO3 (7.5%) solution was added, and the mixture was incubated at 50 °C for 5 min. The absorbance was measured spectrophotometrically at 765 nm. The standard curve was obtained with different concentrations of gallic acid (μg/mL). Unicam Helios UV/Vis spectrophotometer was used to measure the absorbance.

### 3.7.8. Determination of naringin concentration

To investigate the hydrolytic naringinase activity produced by probiotic bacteria, naringenin contents in fermented grapefruit juices were evaluated by HPLC method. The HPLC method was modified according to the method of Ribeiro and Ribeiro (Ribeiro & Ribeiro, 2008) to monitor changes in naringin and naringenin contents. The Surveyor HPLC system (Thermal Scientific Corporation, USA) consisted of a quadruple pump, an autosampler, a photodiode array (PDA) detector, and a column of Supelcosil ™ LC-18 (250 x 4.6 mm, 5 μm) was applied. The mobile phase consisted of acetonitrile (A) and water (B). Separation was performed using a gradient program: 0-8 min 23% A; 8-15 min 23-65% A linear; 15-20 min 65-70% A linear; 20-21 min 70-23% A linear; 21-22 min 23% A. The chromatogram was taken at 280 nm.

The standard solution of naringin (0.1%) was prepared in a mixture of absolute ethanol:sodium acetate buffer 0.02 M, pH 4.0 (1:1) (v:v).

The sample preparation was prepared as follows. Centrifuged fermented grapefruit juices were filtered through Whatman filter and diluted 1:4 (v:v) with sodium acetate buffer 0.02 M, pH 4.0, then were analyzed by HPLC method.

### 3.7.9. Statistical analysis

All experiments were carried out in triplicate. All data are presented as the mean and standard deviation (SD). One-way analysis of variance (ANOVA), unpaired and paired Student's t-tests were done using IBM Statistic SPSS 22 software. Generally, only $p < 0.05$ was accepted as statistically different.

# 4. RESULTS AND DISCUSSION

## 4.1. Production of naringinase by probiotic bacteria

### 4.1.1. Screening bacteria strains for naringinase activity

All investigated strains (16 strains of lactic acid bacteria and 20 strains of bifidobacteria) grew well in their typical medium, and most of them exhibited extracellular naringinase activity. The changes of pH and naringinase production are shown in the **Table 4.1**.

**Table 4.1 Naringinase activity of different probiotic bacteria**

| Strains | pH | | Naringinase activity (IU/mL) |
|---|---|---|---|
| | Initial | After 24 h of fermentation | |
| *L. plantarum* 01 | 7.00 | 5.42 | 2.92 |
| *L. plantarum* 299v | - | 5.32 | 2.87 |
| *L. rhamnosus* B01725 | - | 5.29 | 2.61 |
| *L. curvatus* 2770 | - | 5.23 | 1.88 |
| *L. curvatus* 2768 | - | 5.25 | 1.11 |
| ***L. fermentum* D13** | - | **5.26** | **4.27** |
| *L. fermentum* DT41 | - | 5.24 | 2.01 |
| *Lc. lactis* ssp. *cremoris* B02124 | - | 5.36 | 2.66 |
| *Lc. lactis* B02121 | - | 5.29 | 0.00 |
| *L. sanfrancisco* B02234 | - | 5.22 | 1.42 |
| *L. paracasei* ssp. *paracasei* 05 | - | 5.23 | 1.15 |
| *L. acidophilus* La-5 | - | 5.28 | 1.69 |
| *L. casei* 01 | - | 5.30 | 1.27 |
| *L. casei Shirota* | - | 5.27 | 1.98 |
| *L. delbruecki* ssp. *bulgaricus* | - | 5.63 | 1.83 |
| *L. sakei* DSM 20017 | - | 5.26 | 2.60 |
| *B. longum* B6.1 | 6.75 | 3.97 | 2.03 |
| *B. longum* A1.2 | - | 4.01 | 1.66 |
| *B. longum* A4.4 | - | 3.99 | 0.69 |
| *B. longum* A4.6 | | 4.03 | 0.43 |
| ***B. longum* Bb46** | - | **3.87** | **3.70** |
| *B. longum* K630 | - | 4.05 | 0.36 |
| *B. longum* DSM 16603 | - | 4.02 | 0.90 |
| *B. longum* NCAIM 01819 | - | 4.02 | 1.54 |
| *B. longum* ATCC 15707 | - | 3.98 | 0.85 |

| Strains | pH | | Naringinase activity (IU/mL) |
|---|---|---|---|
| | Initial | After 24 h of fermentation | |
| *B. animalis* B4.1 | - | 4.13 | 0.30 |
| *B. animalis* B320 | - | 3.96 | 1.52 |
| *B. bifidum* B7.5 | - | 3.93 | 2.29 |
| *B. breve* B9.15 | - | 4.16 | 1.14 |
| *B. breve* B10.2 | - | 4.10 | 0.97 |
| *B. breve* NCAIM B01802 | - | 4.01 | 0.97 |
| *B. lactis* Bb12 | - | 4.17 | 1.39 |
| *B. angulatum* DSM 20098 | - | 4.05 | 0.30 |
| *B. adolescentis* NCAIM B01822 | - | 4.05 | 0.84 |
| *B. adolescentis* T | - | 4.03 | 0.00 |
| *B. dentium* NCAIM 02023 | - | 4.01 | 0.42 |

Preliminary analysis revealed that all tested lactic acid bacteria gave the highest naringinase activity after 24 hours of fermentation. Among the investigated strains, *L. fermentum* D13 showed the highest naringinase activity (4.27 IU/mL). Two *L. plantarum* strains, 01 and 299v, also gave high naringinase activity after 24 hours with enzyme titers 2.92 IU/mL and 2.87 IU/mL, respectively. In the case of bifidobacteria, the highest naringinase activity (3.46 IU/mL) was produced by *B. longum* Bb46. Some bifidobacteria, such as *B. longum* B6.1, *B. longum* A1.2, B. *longum* NCAIM 01819, *B. animalis* B320, *B. bifidum* B7.5, *B. breve* B9.15 and *B. lactis* Bb12 strains also showed higher naringinase activity, than 1 IU/mL. Avila *et al.* (2009) reported that *L. plantarum* strains produced α-L-rhamnosidase, which splits naringin into prunin and L-rhamnose. Characterization of rhamnosidase from *L. plantarum* even was described by Beekwilder *et al.* (2009). On hydrolysis of naringin, some studies were found in the literature. Extracellular naringinase was reported to be produced by *Streptomycetes* (Caraveo *et al.*, 2014). Puri *et al.* (2010) produced naringinase using *Staphylococcus xylosus* MAK2, and they also optimized the fermentation medium for the improvement of naringinase production. Another bacterium, namely *Serratia* sp., was isolated from soil and had the ability to produce naringinase (Pavithra *et al.*, 2012) with the maximum enzyme titer of 9.2 U/L. Mukund *et al.* (2014) found five strains that were able to degrade naringin, thus they were positive for extracellular naringinase activity. One (*Bacillus methylotrophicus* CBMB2*)* of them showed the highest activity (3.44 U/L). In all studies, the naringinases from both fungi and bacteria were found in fermentation broths, thus they are extracellular enzymes. Most known sources of naringinase are from fungi including

*Aspergillus niger* VB07 (Kumar *et al.*, 2010), *Aspergillus niger* MTCC 1344 (Puri *et al.*, 2005), *Aspergillus oryzae* 11250 (Zhu *et al.*, 2017a). Recently, an intracellular naringinase produced by *Bacillus amyloliquefaciens* 11568, isolated from soil, was reported (Zhu *et al.*, 2017b).

### 4.1.2. Effect of inoculum ratio

In submerged fermentation, the amount of inoculum is one of the most important factors in growth of microorganisms as well as enzyme production, thus determination of optimum inoculum ratio for maximization of naringinase production was one of my tasks. Different ratios of inoculum (from 2% (v/v) to 8% (v/v) of *L. fermentum* D13 ($10^9$ cfu/mL) were transferred into the fermentation medium for the production of naringinase.

There was a significant effect on naringinase activity of inoculum sizes. Increase in the inoculum size from 2% (v/v) to 4% (v/v) had positive effect on naringinase production. After 24 hours of fermentation, the highest yield of naringinase observed at inoculum size of 4% (v/v) (**Fig. 4.1**). The higher inoculum size (8%) was found to reduce the production of naringinase more than if the lower inoculum size was used (2%). Different optimum inoculum sizes were found by other researchers for different bacteria, 5% (v/v) for *Staphylococcus xylosus* MAK2 (Puri *et al.*, 2011a) and 6% (v/v) for *Micrococcus sp.* (Kumar *et al.*, 2015). Therefore, high inoculum sizes do not necessarily give higher naringinase yield. With the increase in inoculum ratio, the production of naringinase declined due to exhaustion of nutrients in the fermentation medium resulting in decreased metabolic activity of the organism. At the optimum inoculum size, the balance between proliferating biomass and availability of nutrients supported production of the enzyme. The low population of bacteria in the initial fermentation medium can lead to reduced levels of secreted naringinase. This result was not only in agreement with production of enzymes by bacteria but also by fungi (Uyar & Baysal, 2004; Haq *et al.*, 2010; Bansal *et al.*, 2012).

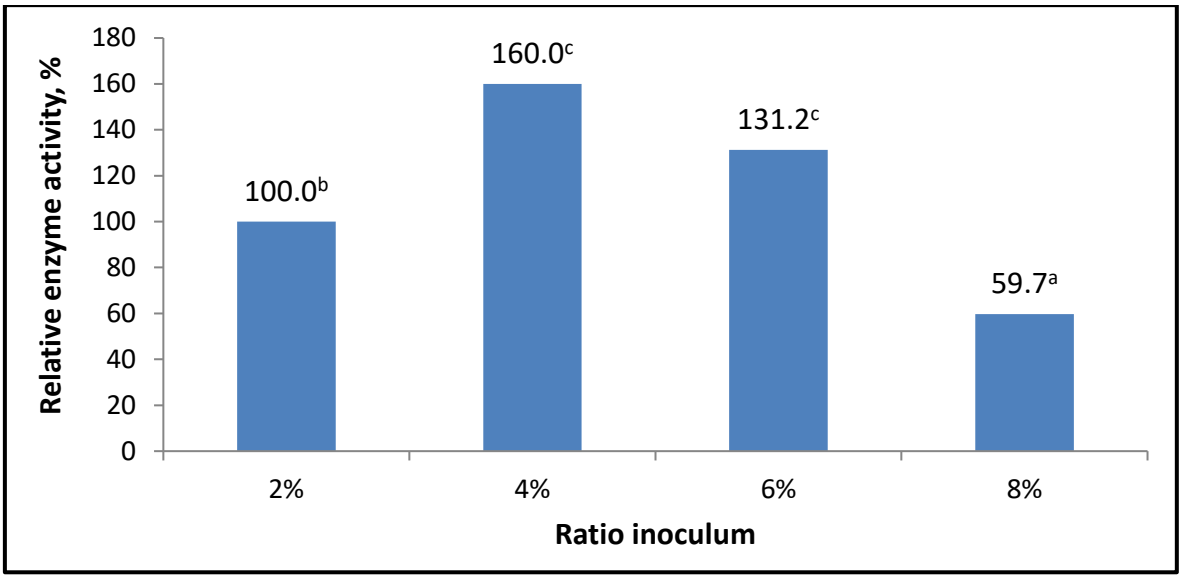


**Figure 4.1 Effect of inoculum ratio on naringinase activity**
[a,b,c] significant difference according to the Tukey's test ($p<0.05$)

### 4.1.3. Influence of pH on production of naringinase

The naringinase activity by *L. fermentum* D13 at varying pH levels showed that the relative enzyme activity increased from pH 5.0 to 6.0. Further increase in pH from 6.0 to 7.0 and 8.0 resulted in decrease of activity of naringinase (**Fig. 4.2**). However, statistical analysis of one-way ANOVA revealed that there was no significant difference in naringinase activities among pH 5.0, 6.0, and 7.0; and among pH 7.0 and 8.0. Between pH 5.0, 6.0 and 8.0, there were significant differences on naringinase activity by *L. fermentum* D13. When the pH of the initial medium was alkaline, there was a decrease in enzyme naringinase activity. Our results are in close agreement with the previous reports studying naringinase production by bacteria. The highest naringinase activity by *Staphylococcus xylosus* MAK2 was obtained when the pH of the medium was 5.5 (Puri *et al.*, 2011a), or initial pH of medium for naringinase production by *Micrococcus sp.* was 6.0 (Kumar *et al.*, 2015).

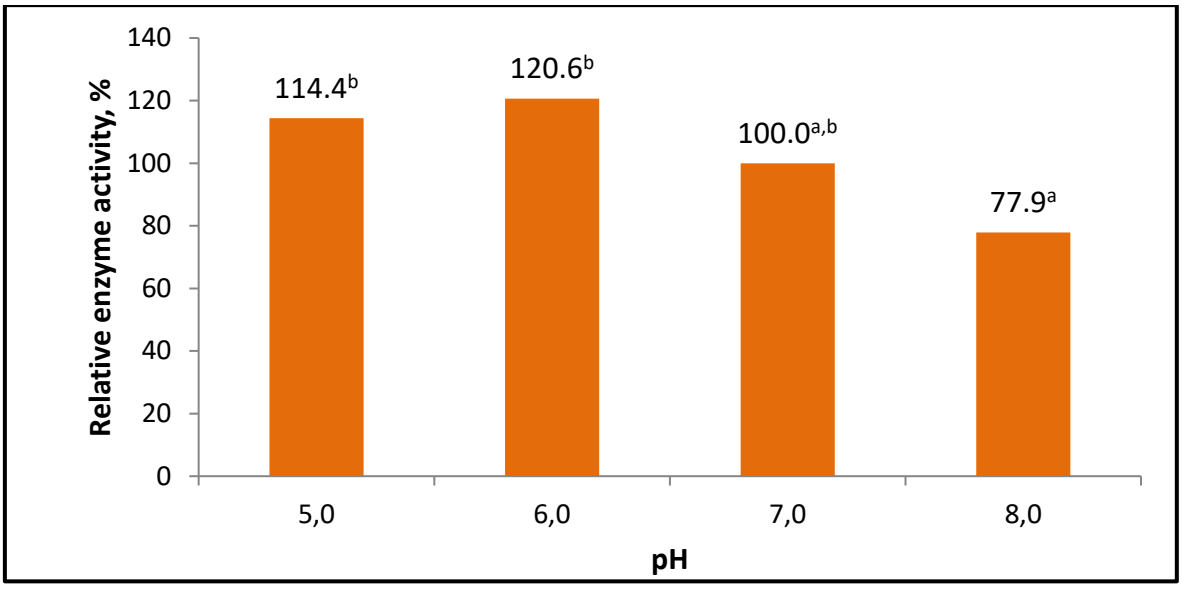


**Figure 4.2 Initial pH of the medium affects naringinase production**
[a,b] significant difference according to the Tukey's test ($p<0.05$)

### 4.1.4. Effect of naringin concentration

The presence and concentration of inducer may play an important role in activation of the encoding gene, thus in enzyme synthesis. In my work, naringin substrate was used as inducer for production of naringinase by *L. fermentum* D13 strain. Adding different naringin concentrations to the fermentation medium, affected naringinase production. *L. fermentum* D13 grew well in naringin modified medium even at high concentration (0.2% w/v). The control naringin concentration was at 0.05%, the optimized naringin concentration at 0.1% (w/v) induced the highest relative enzyme activity (**Fig. 4.3**). According to the Tukey's test ($p<0.05$), there were significant differences in naringinase activity between naringin concentration 0.1% and other concentrations studied. Increase in the naringin concentration did not result higher naringinase activity. Similar results were observed for naringinase production by *A. niger* or *Penicillium* sp. Among inducers naringin, naringenin, rutin and hesperidin, naringin induced the highest naringinase activity by *A. niger* VB07 at the concentration of 0.1% (w/v) (Kumar *et al.*, 2010). Using *A. niger* MTCC 1344 for production of naringinase in solid-state fermentation, in medium supplemented with 10% naringin, also caused maximum induction (Shanmugaprakash *et al.*, 2011). Higher naringinase levels could be obtained by adding citrus peel powder as an inducer, which is rich in naringin (Puri *et al.*, 2011a).

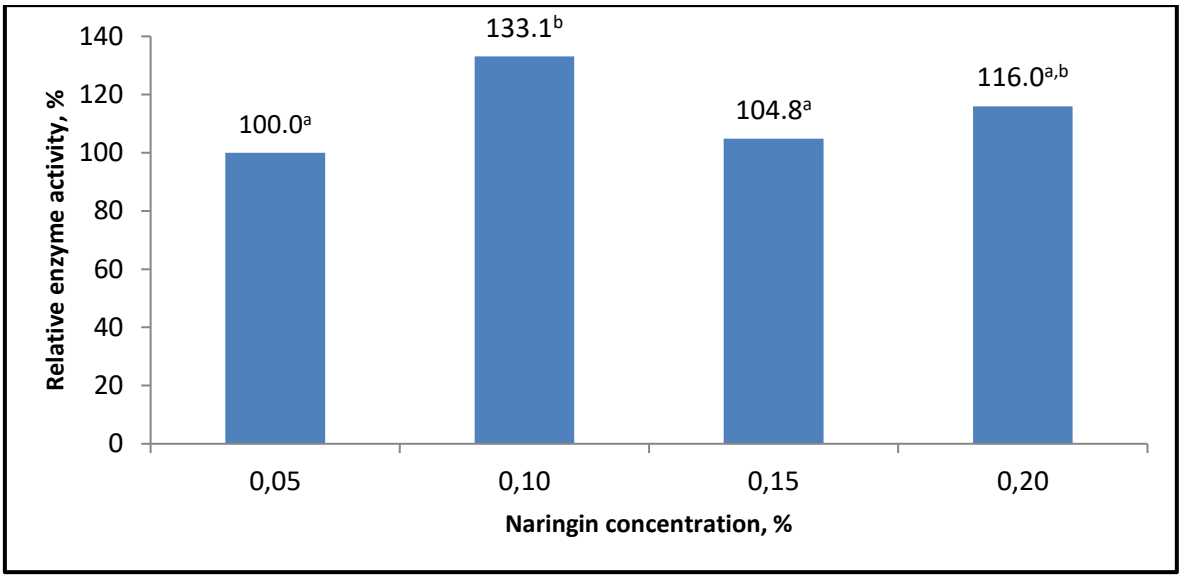


**Figure 4.3 Effect of naringin concentration on production of naringinase by *L. fermentum* D13**

[a,b] significant difference according to the Tukey's test ($p<0.05$)

### 4.1.5. Effect of various carbohydrate sources on naringinase activity

Effect of various main carbon sources (glucose, starch, rhamnose, lactose, maltose, sucrose, fructose, molasses and galactose) on production of naringinase was investigated. The fermentation media were prepared by substitution of glucose in MRS medium with the given carbohydrate. The results are summarized in **Table 4.2** and **Fig. 4.4**.

The highest relative enzyme activity was obtained when *L. fermentum* D13 strain was grown in the medium containing 1% (w/v) sucrose. Other carbohydrates such as molasses, glucose, lactose and galactose also were good carbon sources for naringinase production. Rhamnose and maltose were not suitable substrates for production of naringinase by *L. fermentum* D13 strain, as very low enzyme activities were measured in the fermentation broth after 24 h fermentation. Similar results could be observed in the study of Puri *et al.* (2011a), sucrose and molasses showed the highest increase in naringinase production from *S. xylosus MAK2*. Carbon sources also affected naringinase production of different microorganisms. Production of naringinase by *Serratia* sp. was enhanced in the presence of glucose (Pavithra *et al.*, 2012). In another study, rhamnose and molasses exhibited highest naringinase activity produced by *Aspergillus niger* VB07 after 7 days of fermentation (Kumar *et al.*, 2010).

In the cases of starch, rhamnose, and lactose, the pH values of the media after 24 hours fermentation were pH 5.89, pH 5.81 and pH 5.67, respectively, meaning *L. fermentum D13* strain grew poorly in the medium with these carbohydrates as main carbon sources. This characteristic also was described by (Holzapfel & Wood, 2012).

**Table 4.2 Effect of different carbohydrate sources on pH of medium during fermentation**

| Carbohydrates 1% (w/v) | pH | |
|---|---|---|
| | Initial | After 24 h of fermentation |
| Glucose | 6.08 | 4.32 ± 0.01 |
| Starch | 6.08 | 5.89 ± 0.00 |
| Rhamnose | 6.08 | 5.81 ± 0.00 |
| Lactose | 6.08 | 5.67 ± 0.02 |
| Maltose | 6.08 | 4.9 ± 0.02 |
| Sucrose | 6.08 | 4.21 ± 0.02 |
| Fructose | 6.08 | 4.26 ± 0.03 |
| Molasses | 6.08 | 4.8 ± 0.01 |
| Galactose | 6.08 | 4.32 ± 0.04 |

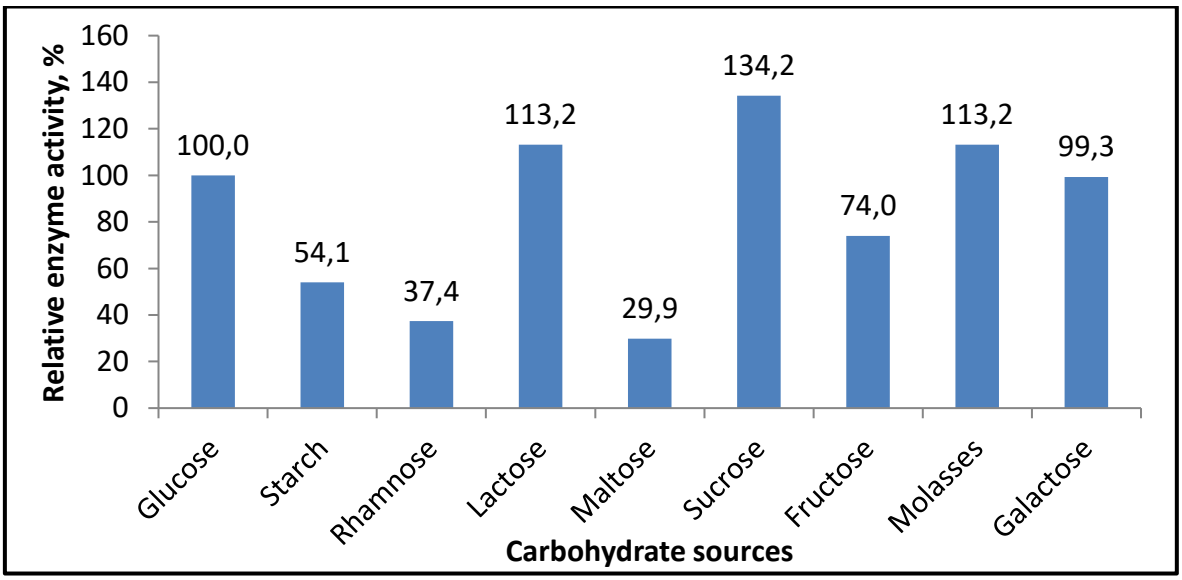


**Figure 4.4 Effect of different carbohydrate sources on naringinase production**

### 4.1.6. Effect of sucrose concentration on naringinase production

In the previous experiment, sucrose showed the highest influence on naringinase production. The effect of sucrose concentration on enzyme production was conducted to find the optimal fermentation medium for naringinase production. The initial sucrose content was varied from 5 to 20 g/L. The results are shown in **Fig. 4.5**.

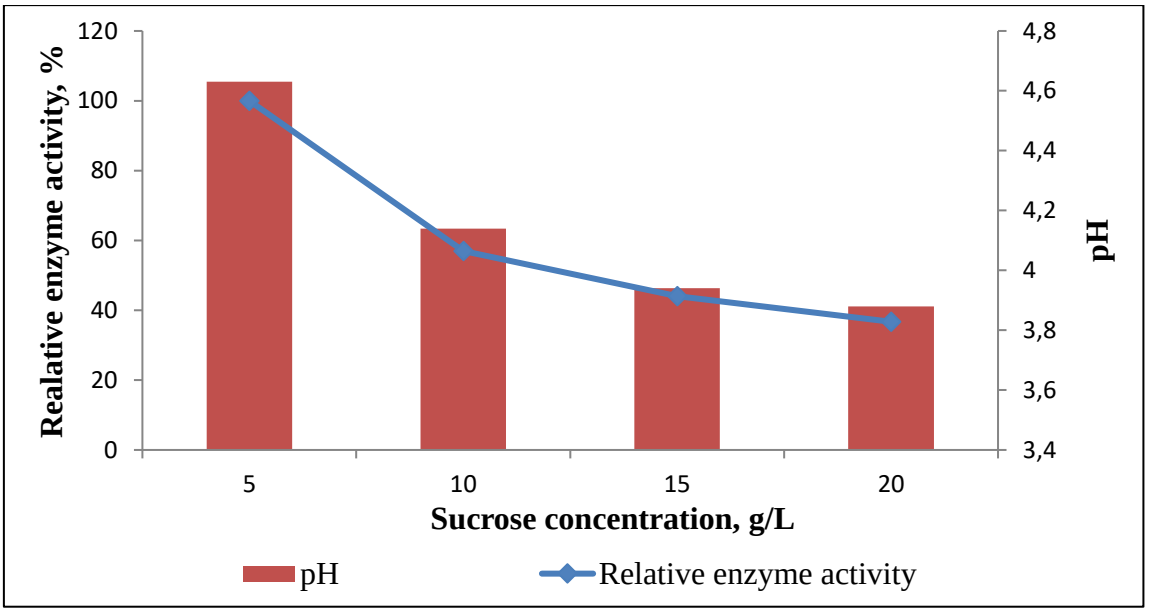


**Figure 4.5 Effect of sucrose concentration on naringinase production**

There was a suppression of naringinase production at the sucrose concentrations of 15 g/L and 20 g/L after 24 h fermentation. The maximal relative enzyme activity was obtained at 5 g/L sucrose concentration. The higher the sucrose content, the higher the growth of *L. fermentum* D13 was, that was also reflected by the pH values. In the study of Puri *et al.* (2011), different concentrations of sucrose (5-30 g/L) were used in the medium to determine the optimum concentration for naringinase production. The highest naringinase yield was observed at the concentration of sucrose 15 g/L.

### 4.1.7. Effect of metal ions on naringinase production

Metal ions play important roles in hydrolysis reactions done by enzymes. The effect of metal ions on naringinase production is shown in **Fig. 4.6**. While $Cu^{2+}$ and $Zn^{2+}$ stimulated naringinase synthesis, the other metal ions ($Fe^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$) inhibited naringinase activity at concentration of 5 mM. The maximum naringinase yield observed when adding $Cu^{2+}$ into the MRS fermentation medium. This result is different from the findings of Puri *et al.* (2011a) and Miake *et al.* (2000), who found that $Ca^{2+}$ has a stimulatory effect on naringinase and α-L-rhamnosidase production.

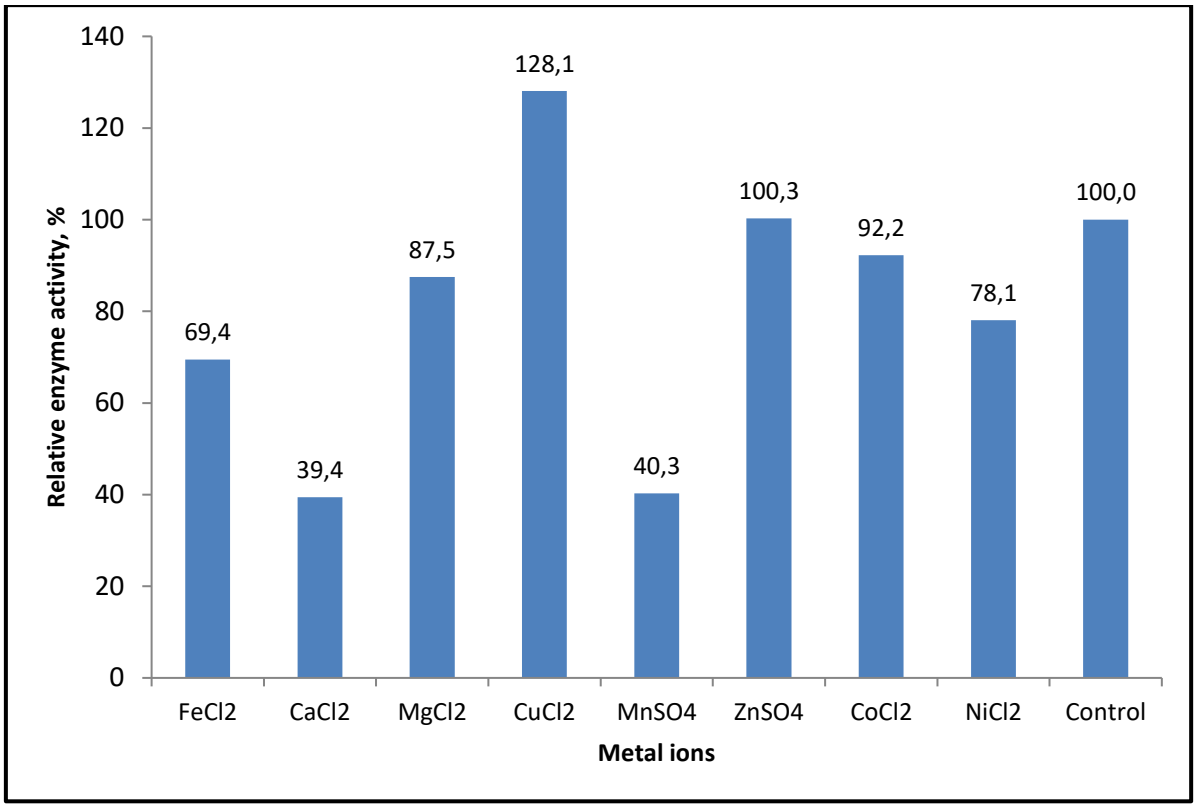


**Figure 4.6 Effect of metal ions on naringinase production by *L. fermentum* D13**

### 4.1.8. Optimization of some fermentation factors for naringinase production

Based on preliminary experiments, three factors, namely pH, naringin, and sucrose, were selected for optimization of naringinase production by *L. fermentum* D13 strain. Central Composite Design (CCD) method with experimental set consisting of 17 runs was applied to determine the optimal levels of these factors. The design of the experiment and results of naringinase activity and biomass production are shown in **Table 4.3.**

Full second order polynomial model was applied to describe the changes of naringinase activity and biomass. The fit of model to the experimental results was first checked by analysis of variance (ANOVA), and results are summarized in **Table 4.4**.

The fisher F-test with a low probability value ($P>F$=0.006) demonstrates a high significantly for the regression model. The goodness of fit of the model was checked by the determination coefficient ($R^2$). In this experiment, the value of determination coefficient was $R^2$ = 0.912, meaning that only 8.8% of the total variations are not explained by the model. The application of RSM with CCD was fitted with a second order polynomial equation. The regression equation obtained after the analysis of variance, is presented as a function of pH, naringin content, and sucrose concentration. The model obtained in terms of the coded factor is:

$$Y = 4.532 + 0.272X_1 + 0.185X_2 + 0.269X_3 - 0.394X_1^2 - 0.352X_2^2 - 0.282X_3^3$$

Where Y is the response, that is naringinase activity (IU/mL), and $X_1$, $X_2$, $X_3$ are the coded values of pH, naringin content (%) and sucrose concentration (g/L), respectively.

**Table 4.3 Design of RSM experiments and results of naringinase and biomass production**

| No | pH | Naringin (%) | Sucrose (g/L) | Naringinase activity (IU/mL) | Biomass (g/L) |
|---|---|---|---|---|---|
| 1 | 5.5 (-1) | 0.05 (-1) | 2.5 (-1) | 2.2 | 1.88 |
| 2 | 6.5 (+1) | 0.05 (-1) | 2.5 (-1) | 3.61 | 2.38 |
| 3 | 5.5 (-1) | 0.15 (+1) | 2.5 (-1) | 3.30 | 1.57 |
| 4 | 6.5 (+1) | 0.15 (+1) | 2.5 (-1) | 3.59 | 2.05 |
| 5 | 5.5 (-1) | 0.05 (-1) | 7.5 (+1) | 3.32 | 3.78 |
| 6 | 6.5 (+1) | 0.05 (-1) | 7.5 (+1) | 3.93 | 4.05 |
| 7 | 5.5 (-1) | 0.15 (+1) | 7.5 (+1) | 3.75 | 3.85 |
| 8 | 6.5 (+1) | 0.15 (+1) | 7.5 (+1) | 3.65 | 4.18 |
| 9 | 5.159 (-1.68) | 0.1 (0) | 5 (0) | 3.09 | 2.85 |
| 10 | 6.84 (+1.68) | 0.1 (0) | 5 (0) | 3.99 | 3.32 |
| 11 | 6 (0) | 0.0159 (-1.68) | 5 (0) | 3.27 | 3.28 |
| 12 | 6 (0) | 0.1841 (+1.68) | 5 (0) | 4.05 | 3.17 |
| 13 | 6 (0) | 0.1 (0) | 0.795 (-1.68) | 3.34 | 1.58 |
| 14 | 6 (0) | 0.1 (0) | 9.205 (+1.68) | 4.37 | 4.32 |
| 15 | 6 (0) | 0.1 (0) | 5 (0) | 4.92 | 3.28 |
| 16 | 6 (0) | 0.1 (0) | 5 (0) | 4.55 | 3.29 |
| 17 | 6 (0) | 0.1 (0) | 5 (0) | 4.08 | 3.22 |

**Table 4.4 Analysis of variance (ANOVA) for the factorial design**

| Sources of variation | Sum of squares | Degrees of freedom | Mean square | *F* value | P value (*Pr* > *F*) |
|---|---|---|---|---|---|
| Regression | 5.77414 | 9 | 0.641571 | 8.04998 | 0.006 |
| Residual | 0.55789 | 7 | 0.079699 | | |
| Total | 6.33203 | 16 | 0.395752 | | |

$R^2$ = 0.912; $R^2$ adjust = 0.799

**Table 4.5 Model coefficients estimated by regression analysis**

| *Factor* | *Coefficient* | *Standard error* | *P-value* |
|---|---|---|---|
| Intercept | 4.53217 | 0.162675 | 1.97E-08 |
| $X_1$ | 0.272402 | 0.0763884 | 0.009146 |
| $X_2$ | 0.185033 | 0.0763885 | 0.045939 |
| $X_3$ | 0.268559 | 0.0763884 | 0.009784 |
| $X_1*X_1$ | -0.393983 | 0.0840675 | 0.002244 |
| $X_2*X_2$ | -0.352274 | 0.0840675 | 0.004084 |
| $X_3*X_3$ | -0.281757 | 0.0840675 | 0.012225 |
| $X_1*X_2$ | -0.2295 | 0.0998114 | **0.055045** |
| $X_1*X_3$ | -0.14925 | 0.0998114 | **0.178484** |
| $X_2*X_3$ | -0.1165 | 0.0998114 | **0.281346** |

Significant at $P < 0.05$

The optimum values of pH, naringin and sucrose concentration for optimization of naringinase production were 6.1, 0.1% (w/v) and 6.0 g/L, respectively (**Fig. 4.7**). These results are different from those reported by Puri *et al.* (2010), where the optimization of medium components for naringinase production from *Staphylococcus xylosus* MAK2 was also investigated (Puri *et al.*, 2010). In this study, a second-order polynomial was derived by multiple regression analysis on the Sources of carbon (sucrose) and nitrogen (sodium nitrate), as well as an inducer (naringin) and pH levels. Using response surface methodology applying CCD, the optimum values for the critical components were obtained as follows: sucrose 10.0%; sodium nitrate 10.0%; pH 5.6; biomass concentration 1.58%; and naringin 0.50 (w/v) %. Under optimal conditions, the experimental naringinase production was 8.45 IU/mL. These differences can be explained by the different microorganisms used.

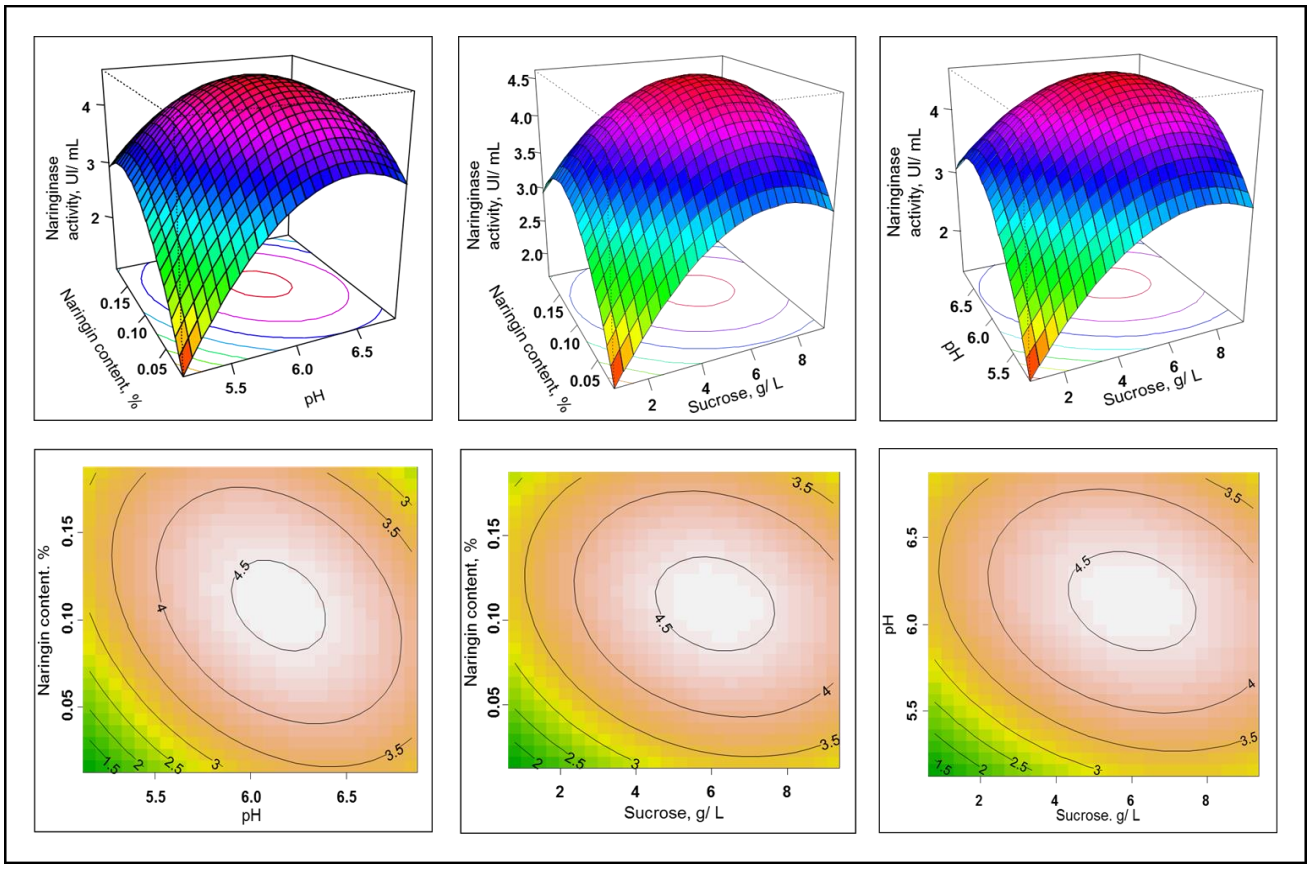


**Figure 4.7 Response surface and counter plot of the model showing the effect of pH, naringin content and sucrose content on production of naringinase from *L. fermentum* D13**

## 4.2. Characterization of crude naringinase

### 4.2.1. Effect of pH on naringinase activity

Changes of naringinase activity values against pH are shown in the **Fig. 4.8**. The enzyme showed maximum activity at pH 4.5. It was still relatively active at pH 4.0, having lost only 16% of its maximum activity. At pH 3.6 and pH 5.0, its activity was about 70% of the maximum activity. The activity of the enzyme decreased dramatically above pH 5.5. The optimum pH for the hydrolysis of naringin by *L. fermentum* D13 naringinase was 4.5 at 40 °C.

These findings are in agreement with those reported for naringinase from other sources. Values of the optimal pH for naringinase activity from various sources range from pH 4.0 to pH 7.5. Most fungal naringinases, such as from *P. ulaisen* (Rajal *et al.*, 2009), *A. niger* (Ni *et al.*, 2012a), *A. aculeatus* JMUdb058 (Chen *et al.*, 2013), and *A. oryzae* 11250 (Zhu *et al.*, 2017a), had been reported to have optimum pH from 4.0 to 5.0 (**Table 2.2**). Naringinase activity of α-L-rhamnosidase from bacteria *L. plantarum* NCC245 (Avila *et al.*, 2009), *Pediococcus acidilaciti* DSM 20184 (Michlmayr *et al.*, 2011), and *Cryptococcus albidus* (Borzova *et al.*, 2018) had pH optima at 5.0, 4.5-5.0 and 5.0, respectively. A broad range of pH optimum is preferable for applications of naringinase in food, especially in beverage industry.

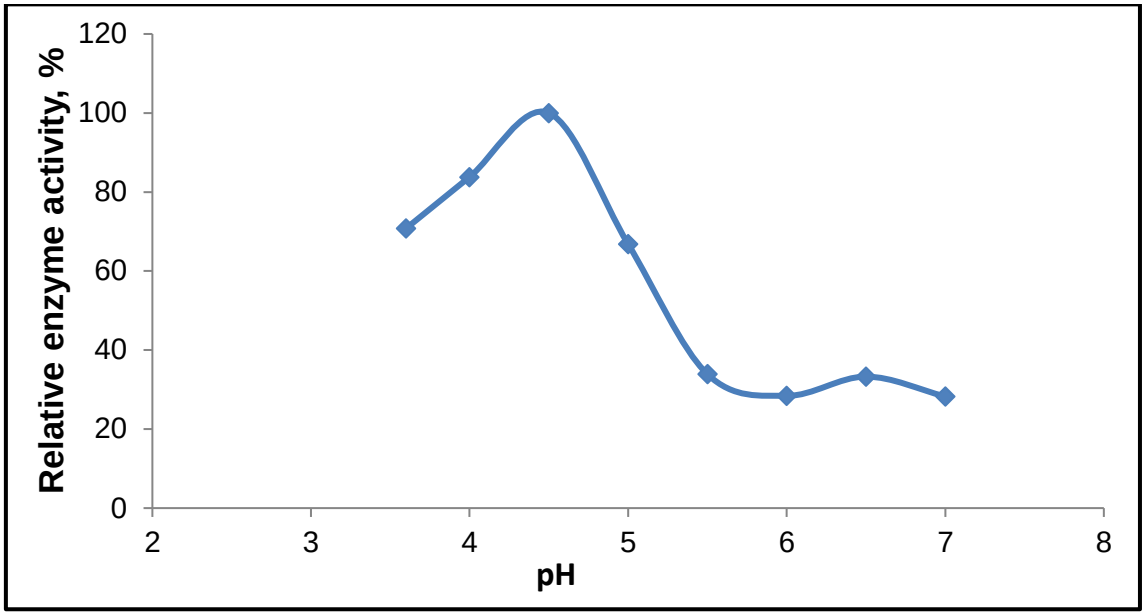


**Figure 4.8 Effect of pH on the activity of naringinase (at temperature 40 °C)**

### 4.2.2. Effect of temperature on naringinase activity

The effect of temperature on naringinase activity is demonstrated in **Fig. 4.9**. The optimum temperature for the crude enzyme at pH 4.0 was found to be 40 °C. The activity of enzyme lost about 15% of its maximum activity at 45 °C. Only 50% of the maximum activity was observed at 30 °C, 50 °C and 55 °C.

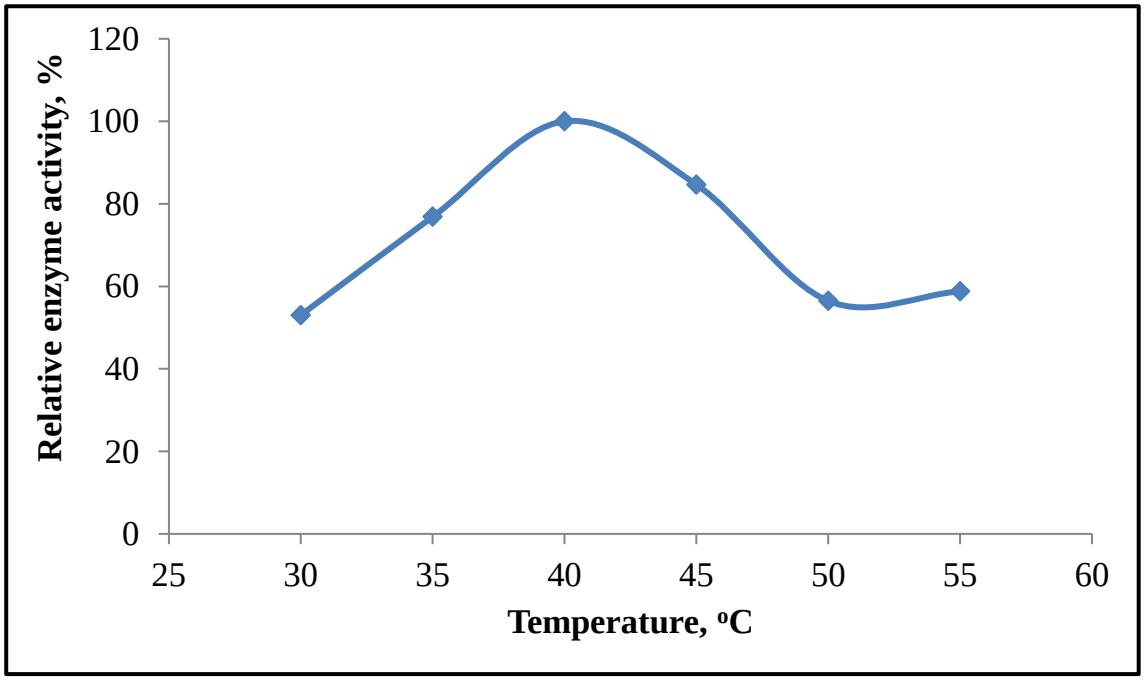


**Figure 4.9 Effect of temperature on naringinase activity (at pH 4)**

This result was correlated with the study conducted previously using other strains. The optimal temperature for the activity of the naringinase as well as α-L-rhamnosidase from various species of microorganism has been obtained to range from 40 °C to 60 °C (**Table 2.2**). Naringinase from *Aspergillus* strains, *A. kawachi* (Koseki *et al.*, 2008), *A. brasiliensis* MTCC1344 (Shanmugaprakash *et al.*, 2014), and *A . oryzae* 11250 (Zhu *et al.*, 2017a) showed maximum activity at 50 °C, 60 °C, and 45 °C, respectively. The higher temperature 55 °C, 60 °C, and 57 °C were optimal for the activity of the naringinase from *P. decumbens* PTCC 5248 (Norouzian *et al.*, 2000) and of α-L-rhamnosidase from *P. ulaiense* (Rajal *et al.*, 2009), and *P . corylopholum* MTCC-2011 (Yadav *et al.*, 2013), respectively. Both enzyme naringinase from bacterial sources *Pseudomonas paucimobilis* FP2001 (Miake *et al.*, 2000) and *Bacillus amyloliquefaciens* 11568 (Zhu *et al.*, 2017b) exhibited good activity at 45 °C.

### 4.2.3. Effect of different metal ions on naringinase activity

The influence of different metal ions present in the reaction mixture at 5 mM concentrations on activity of naringinase is shown in **Fig. 4.10**. The presence of 5 mM $Cu^{2+}$ had a significant positive effect on the enzymatic activity. It is interesting that this result is similar to the result of the experiment on checking the effect of metal ions present in fermentation medium on naringinase production by *L. fermentum* D13. No effect was observed in the case of $K^{+}$ on the naringinase activity. The crude naringinase was inhibited strongly (about 50% reduction of activity) in the case of 5mM $Al^{3+}$. The enzyme activity was slightly restricted by $Ca^{2+}$ and $Mn^{2+}$.

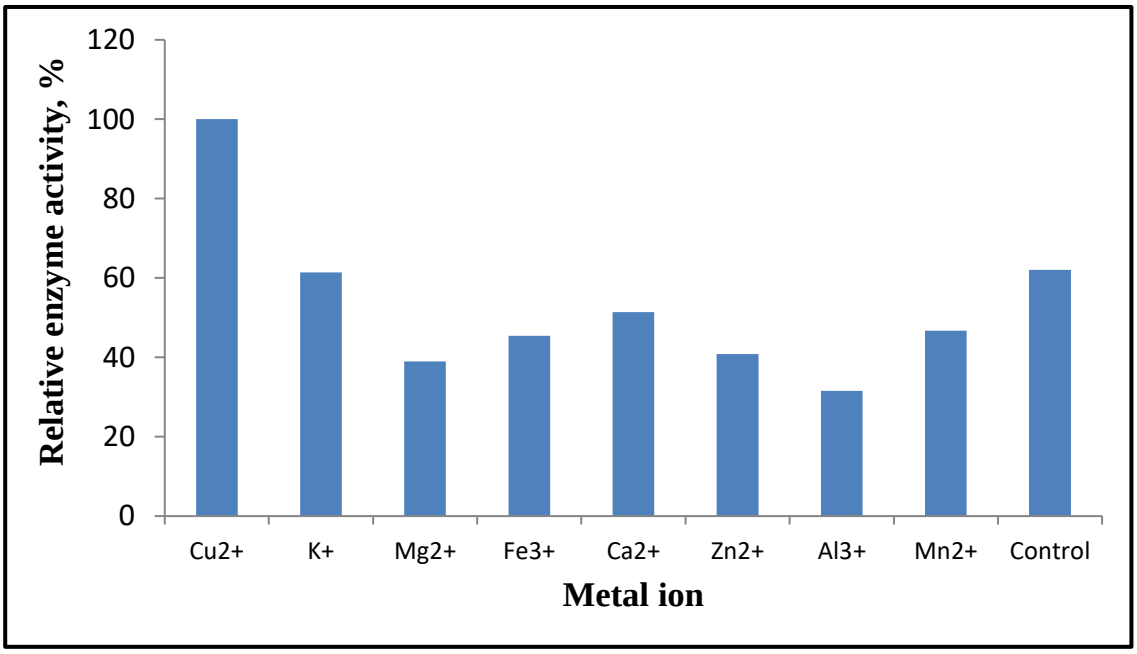


**Figure 4.10 Effect of metal ions on activity of naringinase**

There are different results from other authors on the effect of metal ions on naringinase as well as α-L-rhamnosidase activities. Presence of $Hg^{2+}$, $Ag^{+}$, $Cu^{2+}$ and $Mn^{2+}$ showed inhibiting effect on the enzymatic activity from *P. ulaiense* (Rajal *et al.*, 2009), *A. oryzae* 11250 (Zhu *et al.*, 2017a), *L. plantarum* NCC245 (Avila *et al.*, 2009), and *A. niger* (Puri & Kalra, 2005). In most of cases, $Ca^{2+}$ and $Co^{2+}$ are considered as simulators to the activity of naringinase from *A. terreus* (Gallego *et al.*, 2001), *L. plantarum* NCC245 (Avila *et al.*, 2009) and *A. niger* 1344 (Puri & Kalra, 2005). The difference between our results and other ones could be explained by the different source of the enzyme.

## 4.3. Application experiments

Naringinase from probiotic bacteria was studied in simultaneously fermentation and debittering of grapefruit juice.

### 4.3.1. Viability of probiotic microorganisms

❖ ***Fermentation with monoculture***

Grapefruit juice (pH 6.3) was fermented by monoculture of *L. plantarum* 01, *L. rhamnosus* B01725, *L. fermentum* D13 and *Bifidobacterium bifidum* B7.5. The initial cell concentration was adjusted to about $10^6$ cfu/mL. Fermentation process was carried out at 37 °C for 24 hours, followed by refrigerated storage at 4 °C for 12 weeks.

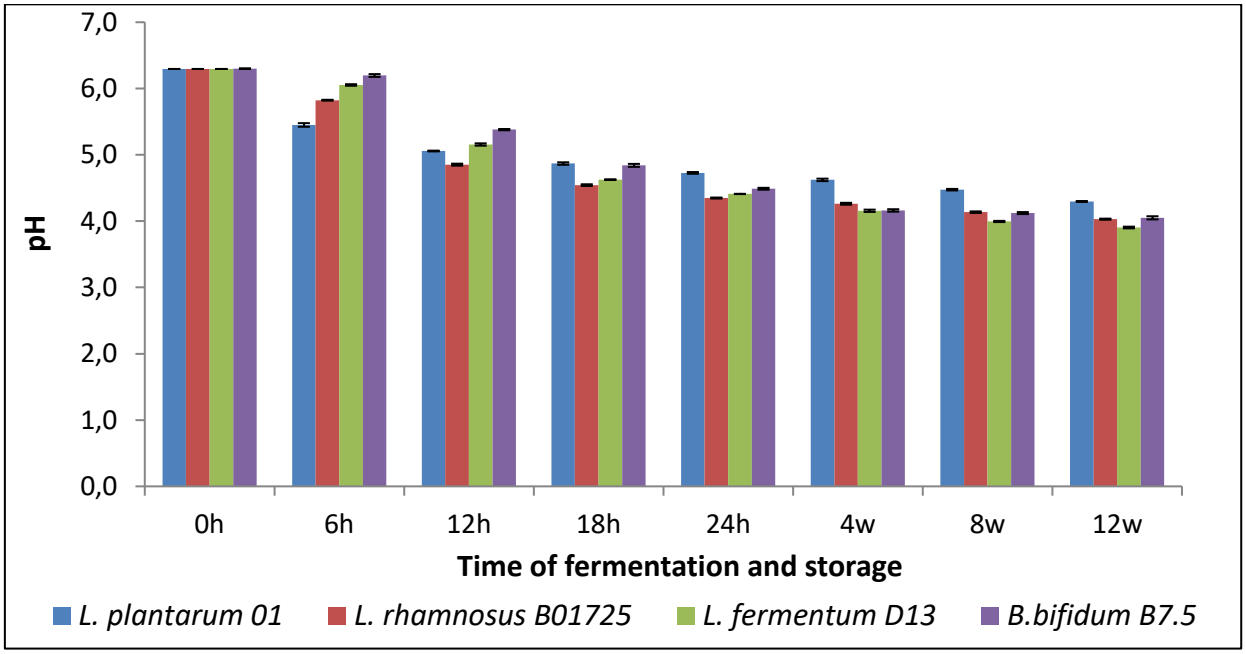


**Figure 4.11 Changes of pH of grapefruit juice during fermentation and storage by monocultures: *L. plantarum* 01, *L. rhamnosus* B01725, *L. fermentum* D13 *and B. bifidum* B7.5**

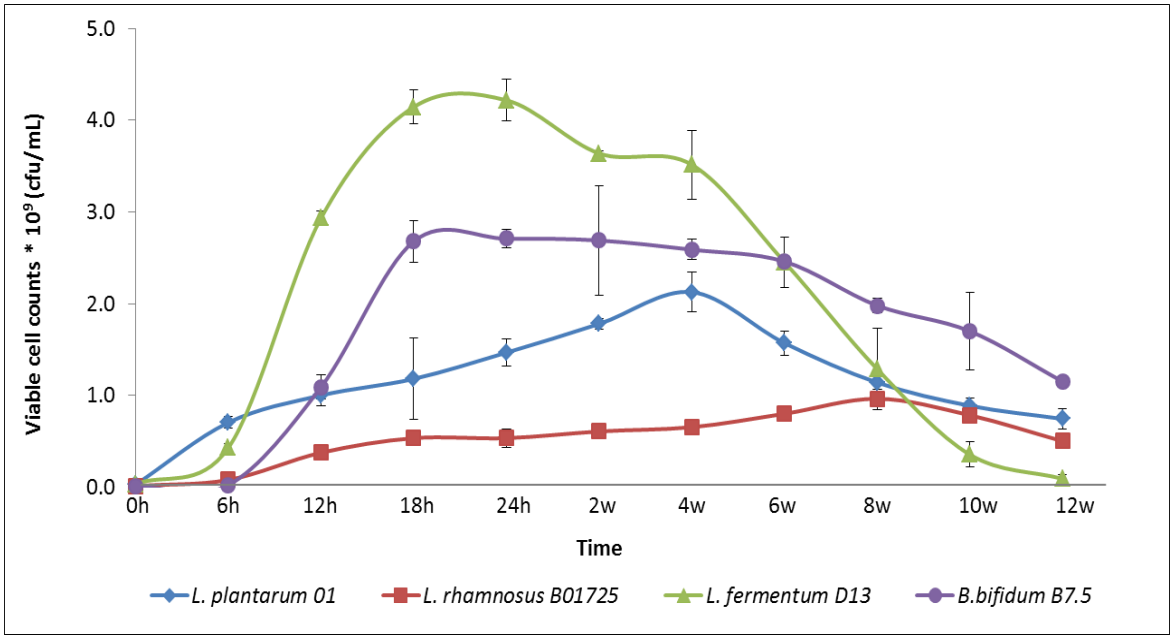


**Figure 4.12 Change of cell numbers of *L. plantarum* 01, *L. rhamnosus* B01725, *L. fermentum* D13 *and B. bifidum* B7.5 during fermentation and storage**

All investigated lactic acid bacteria were showed to be capable of growing well in sterilized grapefruit juice without nutrition supplementation. They were not only able to survive but also to utilize the carbohydrates in grapefruit juice for their cell synthesis and metabolism. The pH of fermented juices decreased with different rates for different cultures during fermentation (**Fig. 4.11**). The pH of grapefruit juice fermented by *L. plantarum* 01 strain had a dramatic decrease in the first 6 h of fermentation (from pH 6.3 to pH 5.45), and then felt steadily to pH 4.87. In the case of *L. fermentum* D13 and *B. bifidum* B7.5 strains, the pH of grapefruit juices decreased slightly in the first 6 h (pH 6.05 and 6.2, respectively) before sharply falling in the next 18 h of fermentation. A significant decrease of pH of grapefruit juice fermented by *L. rhamnosus* B01725 strain was observed in the first 12 h of fermentation (pH 4.85), after that the pH decreased slightly. At the end of the fermentation process, the pH ranged from 4.35 to 4.73. During storage at 4 °C for 12 weeks, the pH of the fermented grapefruit juice decreased to around pH 4.0. The lowest pH (3.90) was recorded in juiced fermented by *L. fermentum* D13 strain. In case of *L. plantarum* 01 strain, pH value was 4.3 at week 12 of storage. The reduction of pH during fermentation and storage of probiotic products is of great importance for maintaining the quality of the product as well as the quality of the end product.

The results of survivability of investigated strains during fermentation and 12 weeks of storage at 4 °C are presented in **Fig. 4.12**. In all cases, cell counts were higher than $10^8$ cfu/mL after 24 h of fermentation with values, in the cases of *L. fermentum* D13, *L. plantarum* 01 and

*B. bifidum* B7.5, of $4.22\times10^9$ cfu/mL, $2.71\times10^9$ cfu/mL and $1.47\times10^9$ cfu/mL, respectively. The volumetric productivities corresponded to $17.5\times10^{10}$ cfu/L*h, $11.2\times10^{10}$ cfu/L*h, $6.13\times10^{10}$ cfu/L*h, respectively. Compared to the data obtained for fermentation of carrot juice with *Bifidobacterium* strains (Kun *et al.*, 2008), the cell yield of *B. bifidum* B7.5 was in agreement with the results of *B. lactis* Bb-12, *B. bifidum* B7.1 and *B. bifidum* B3.2 strains.

During the storage of probiotic grapefruit juice, the viable cell counts of *L. plantarum* 01 increased from $(1.47\pm0.15)\times10^9$ cfu/mL to $(2.13\pm0.22)\times10^9$ cfu/mL at week four of storage. Then, there was a reduction of viable cell counts to $(0.74\pm0.11)\times10^9$ cfu/mL, which is considered a great value for fermented probiotic products (Shah, 2007). The similar trend was observed in the case of *L. rhamnosus* B01725, the viable cell count increased during storage, and reached the highest value at week eight of storage ($0.96\pm0.08\times10^9$ cfu/mL), decreasing thereafter to $(0.5\pm 0.06)\times10^9$ cfu/mL. The results presented herein are in agreement with those presented on evaluating the viability of *L. casei* in probiotic cashew apple juice after 42 days of preservation at 4 °C (Pereira *et al.*, 2011). They reported that the cell number of *L. casei* increased during storage until day 21 of storage at 4 °C before having a slight reduction from day 35. According to the storage assay carried out in the study on fermentation of beet juice by beneficial lactic acid bacteria (Yoon *et al.*, 2005), it was observed that although the lactic cultures in fermented beet juice gradually lost their viability during cold storage, the viable cell counts in the fermented beet juice still remained at $10^6 – 10^8$ cfu/mL after 4 weeks of cold storage at 4 °C.

Both strains *L. fermentum* D13 and *B. bifidum* B7.5 showed decrease in the viable cell counts during storage. After 24 h of fermentation, the viability of *B. bifidum* B7.5 strain was stable around $2.6\text{-}2.7\times10^9$ cfu/mL for 4 weeks of storage before falling slightly to $1.15\times10^9$ cfu/mL. *L. fermentum* D13 strain had the lowest stability during the storage, although it reached the highest cell number ($4.22\times10^9$ cfu/mL) among investigated strains. The cell counts of *L. fermentum* D13 strain began to decrease from week 4 of storage. At the end of storage, the number of surviving cells of *L. fermentum* D13 strain was $9\times10^7$ cfu/mL, which was 5-12 times lower than that of other strains. The results can be explained by the low pH of medium that could lead to the reduction of cell numbers, as the lowest pH (3.90) of fermented grapefruit juice at the end of storage was measured in the case of *L. fermentum* D13 strain. The main reason for the decrease in the viability of probiotic cells is the low pH and high acidity resulting from bacterial growth and fermentation (Zandi & Berenjy, 2016). Factors affecting the viability of probiotic cultures include acidity (pH), oxygen level, lack of nutrients, and presence of antimicrobial substance in the product (Shah, 2001). Overall, grapefruit juice can be a potential growth medium for lactic acid bacteria, and

functional beverage can be produced from grapefruit juice containing high number of probiotic cells after fermentation and maintaining probiotic content during storage.

❖ ***Fermentation with mixed cultures***

The interactions between lactic acid bacteria may stimulate the growth of one or each bacterium in the community. The use of combined cultures of lactobacilli and bifidobacteria also may change the chemical composition and sensorial properties of the fermented fruit juice.

**Table 4.6 Cell numbers (*$10^9$) of bifidobacteria, lactobacilli, and total count of mixed cultures during fermentation and storage**

| **Time** (hour, week) | | 0h | 6h | 12h | 18h | 24h | 2w | 4w | 6w | 10w | 12w |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | | | | | | | | | | | |
| **(1)** | *L. plantarum* 01 | 0.02 | 0.48 | 1.07 | 1.61 | 1.67 | 2.00 | 2.00 | 1.88 | 1.54 | 1.31 |
| | *B. bifidum* B7.5 | 0.00 | 0.57 | 0.99 | 1.77 | 2.18 | 2.54 | 2.48 | 2.15 | 1.54 | 1.25 |
| | **Total** | **0.02** | **1.05** | **2.06** | **3.38** | **3.85** | **4.54** | **4.48** | **4.03** | **3.08** | **2.56** |
| **(2)** | *L. rhamnosus* B01725 | 0.01 | 0.06 | 0.47 | 0.84 | 1.01 | 1.14 | 1.15 | 1.22 | 0.87 | 0.85 |
| | *B. bifidum* B7.5 | 0.00 | 0.06 | 0.47 | 0.87 | 1.06 | 1.14 | 1.17 | 1.34 | 0.76 | 0.58 |
| | **Total** | **0.01** | **0.12** | **0.94** | **1.71** | **2.07** | **2.28** | **2.32** | **2.56** | **1.63** | **1.43** |
| **(3)** | *L. fermentum* D13 | 0.02 | 0.26 | 2.32 | 3.57 | 3.82 | 3.19 | 2.77 | 2.83 | 1.55 | 1.20 |
| | *B. bifidum* B7.5 | 0.00 | 0.26 | 2.39 | 3.43 | 3.44 | 3.22 | 3.06 | 3.08 | 1.67 | 1.25 |
| | **Total** | **0.02** | **0.52** | **4.71** | **7.00** | **7.26** | **6.41** | **5.83** | **5.91** | **3.22** | **2.45** |

**(1), (2), (3):** grapefruit juice fermented by combination of *B. bifidum* B7.5 and *L. plantarum* 01 or *L. rhamnosus* B01725 or *L. fermentum* D13, respectively.

In my work, the grapefruit juice was inoculated with ratio 1:1 of lactobacilli and bifidobacteria. The initial cell counts were $10^6 – 10^7$ cfu/mL for each strain (**Table 4.6)**.

All combinations showed higher total viable counts than monocultures in the fermentation. After 24 h of fermentation, significantly high cell numbers were observed in cases of *L. plantarum* 01 strain and *L. fermentum* D13 strain in combination with *B. bifidum* B7.5 strain, $1.67\times10^9$ and $3.82\times10^9$ cfu/mL, respectively. The similar result had been seen in the mixture of *B. bifidum* B7.5 strain and *L. fermentum* D13 that the cell count of *B. bifidum* B7.5 strain was $3.44\times10^9$ cfu/mL after fermentation. The total colony forming units after fermentation period were $2.07\times10^9$ cfu/mL, $3.85\times10^9$ cfu/mL, $7.26\times10^9$ cfu/mL for the 3 combinations between *B. bifidum* B7.5 and *L. rhamnosus* B01725 or *L. plantarum* 01 or *L. fermentum* D13, in order. In the study conducted by Trontel *et al*. (2011), production of D- and L-lactic acid by mono culture of *Lactobacillus* sp. and mixed culture of *Lactobacillus* sp. and *Lactobacillus amylovorus* DSM $20531^T$ strain was investigated. Maximum viable cell numbers of $8.3\times10^8$ and $1.1\times10^9$ cfu/mL were observed for *Lactobacillus* sp. monoculture and mixed culture, respectively, in MRS medium with glucose after

12 h of cultivation at 40°C (Trontel *et al.*, 2011). Different mixed cultures of lactobacilli and bifidobacteria were used for fermentation of apricot juice. The volumetric productivities of mixed cultures were significantly higher than ones obtained in monoculture fermentation (Bujna *et al.*, 2018). Champagne *et al.* (2010) examined the growth ability of *L. delbrueckii* subsp. *lactis* R0187, *L. helveticus* R0052, *L. rhamnosus* R0011 and *B. longum* R0175 in combination with *Streptococcus thermophilus* cultures in milk and a laboratory soy beverage. They found that there was a symbiosis between *S. thermophilus* and *L. helveticus* or *B. longum* (Champagne *et al.*, 2010). The mixed starter cultures of lactic acid bacteria were evaluated for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures (Gardner *et al.*, 2001). They reported that there were greater differences between the pure cultures than mixed ones with respect to growth in vegetable juice medium and viability during storage.

During the storage period, the total viable cell numbers of the three combinations varied from $1.43 \times 10^9$ cfu/mL to $2.56 \times 10^9$ cfu/mL. The mixtures of *L. bifidum* B7.5 strain and *L. plantarum* 01 strain or *L. fermentum* D13 strain resulted in $2.56 \times 10^9$ cfu/mL and $2.45 \times 10^9$ cfu/mL, respectively, and were significantly higher than of the combination of *L. bifidum* B7.5 strain and *L. rhamnosus* B01725 strain. In the case of *L. fermentum* D13, the number of surviving cells was improved significantly in mixed culture fermentation ($1.2 \times 10^9$ cfu/mL) compared with $9.0 \times 10^7$ cfu/mL in the case of monoculture fermentation at the end of the 12 week-storage, although the pH of fermented grapefruit juice (pH 3.92) also was as low as in monoculture fermentation (pH 3.9) at the end of storage.

Beeren's medium is claimed to be selective for bifidobacteria from faeces of human origin. According to the ratio of lactobacilli and bifidobacteria during mixed culture fermentation and whole storage period is always 1:1, it could be explained that lactobacilli may grew in the Beeren's medium. Or the ratio was also the result of synergistic of mixed cultures. Thus, the growth ability of *L. fermentum*, *L. plantarum*, and *L. rhamnosus* in Beeren's medium should be checked to explain this result.

In most cases, the viable cell concentrations of fermented grape fruit were $10^8$–$10^9$ cfu/mL, which is above the required level of $10^7$ cfu/mL for commercial probiotic products. That makes the obtained fermented grapefruit juice suitable for probiotics delivery.

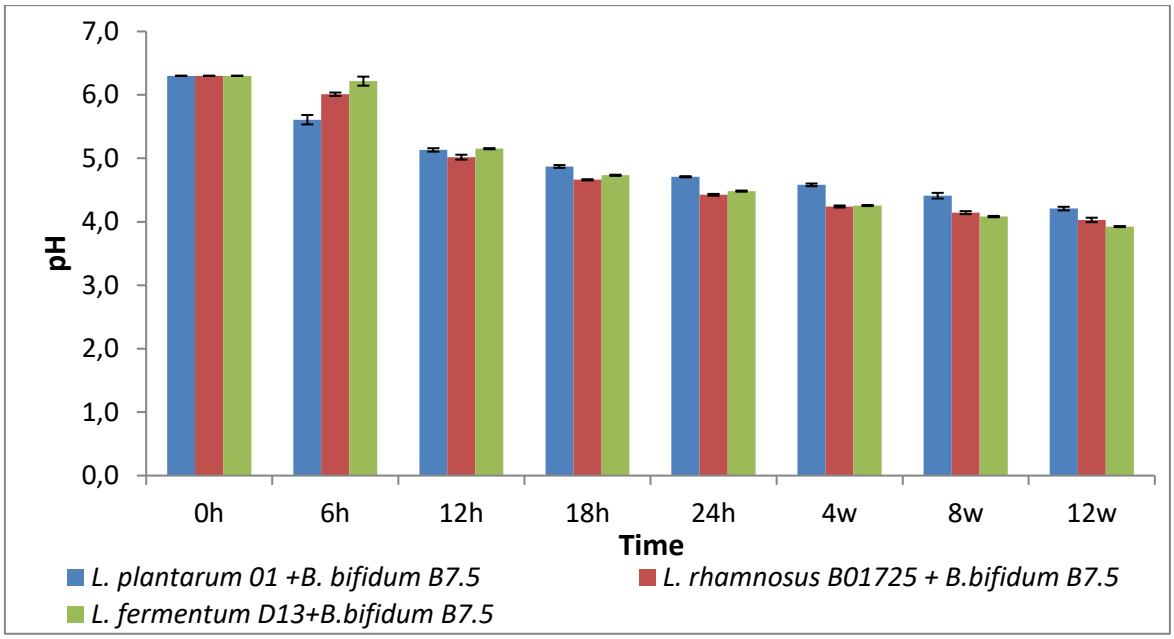


**Figure 4.13 Changes of pH of mixed cultures during fermentation and storage**

The fermentable sugars in grapefruit juice were glucose, fructose, and sucrose with the initial concentration of 3.6, 3.1, and 2.5 g/100 mL, respectively. The pH of juice dropped from 6.3 to about pH 4.4–4.8 by the end of fermentation in both mono and mixed cultures, showing intensive growth and metabolic activity of the probiotic bacteria (**Fig. 4.13**). During the fermentation of grapefruit juice, the concentration of sucrose decreased at the same rate in all cases (from 2.01 g/100 mL to 2.17 g/100 mL). According to the Tukey's test ($p<0.05$), there were no significant differences among the sucrose contents of the fermented grapefruit juices after 24 h of fermentation. The contents of glucose and fructose were utilized at different rates by mono and mixed cultures. Significant utilization of glucose and fructose were observed in grapefruit juice fermented by *L. fermentum* D13 strain (2.31 g/100 mL) and *L. rhamnosus* B01725 strain (2.03 g/100 mL), respectively. The carbohydrates and some organic acid contents of grapefruit juice at the end of fermentation are shown in **Table 4.7**.

The results on consumption of sucrose was in agreement with the study of Bujna *et al.* (2017) on lactic fermentation of apricot juice by mono cultures of probiotic *Lactobacillus* and *Bifidobacterium* strains. Minimum consumption of glucose and maximum of fructose were observed when *L. rhamnosus* B01725 strain was used as monoculture and in combination for fermentation of grapefruit juice. The sugars can be fermented by probiotic bacteria via the Embden-Meyerhof pathway (EMP) or the phosphoketolase pathway (PKP), leading to homolactic and heterolactic fermentation profiles, respectively.

**Table 4.7 Change of carbohydrates during grapefruit juice fermentation**

| Strain | Sucrose | Glucose (g/100 mL) | Fructose |
|---|---|---|---|
| *L. plantarum* 01 | $2.01^{a} \pm 0.03$ | $2.52^{c,d} \pm 0.04$ | $2.61^{a} \pm 0.04$ |
| *L. rhamnosus* B01725 | $2.17^{a} \pm 0.11$ | $3.01^{a} \pm 0.16$ | $2.03^{c} \pm 0.1$ |
| *L. fermentum* D13 | $2.07^{a} \pm 0.02$ | $2.31^{d} \pm 0.02$ | $2.7^{a} \pm 0.02$ |
| *B.bifidum* B7.5 | $2.17^{a} \pm 0.17$ | $2.8^{a,b,c} \pm 0.21$ | $2.29^{b} \pm 0.17$ |
| *L. plantarum* 01 + *B. bifidum* B7.5 | $2.04^{a} \pm 0.04$ | $2.62^{b} \pm 0.05$ | $2.68^{a} \pm 0.06$ |
| *L. rhamnosus* B01725 + *B. bifidum* B7.5 | $2.07^{a} \pm 0.07$ | $2.91^{a,b} \pm 0.09$ | $2.2^{b,c} \pm 0.05$ |
| *L. fermentum* D13+ *B.bifidum* B7.5 | $2.15^{a} \pm 0.03$ | $2.6^{b,c,d} \pm 0.02$ | $2.62^{a} \pm 0.02$ |

Different superscript letters (a-d) in the same column show significant difference according to the Tukey test ($p<0.05$)

The reduction of pH during fermentation of probiotic products is a great importance for maintaining the quality of the product as well as the quality of the end product. The lactic acid and acetic acid were produced in grape fruit juice in the concentration range 9.6 – 190 mM and 8.9 – 1074 mM, respectively (**Table 4.8**). These results were completely higher than those conducted by Champagne and co-workers (2009) when they cultured mono and mixed starter of probiotic strains in milk and a laboratory soy beverage (Champagne *et al.*, 2009). Our results also are better than ones obtained by fermentation of apricot with some LAB and mixed cultures of LAB and bifidobacteria (Bujna *et al.*, 2018). After fermentation process, the highest lactic acid and acetic acid production was observed for the strain *L. plantarum 01* strain (186 mM and 1074 mM, respectively) and the lowest for the strain *B. bifidum* B7.5 (9.6 mM and 8.9 mM, respectively). The lactic acid and acetic acid production were significant difference between *Lactobacillus* and *Bifidobacterium*. In the case of grapefruit juice fermented by *Lactobacillus*, the greater acetic acid production was observed. Especially, the ratios of lactic acids to acetic acids here are disagreement with the results published by the other authors (Kun *et al.*, 2008; Bujna *et al.*, 2018). Our data showed these ratios were 0.17, 0.27 and 0.3 in the case of *L. plantarum* 01, *L. rhamnosus* B01725 and *L. fermentum* D13, respectively. The results can be related to different biochemical pathway (e.g. the degradation product of the produced lactic acid), the citrate metabolism and/or the heterofermentative pathway (Zalán *et al.*, 2009). The presence of citric acid in the grapefruit juice with the initial concentration 123 mM can explain the formation of acetic acid by *Lactobacillus* species through the citric cycle (Krebs cycle) leading to decrease the concentration of citric acid in grapefruit juice and increase the acetic acid content (Starrenburg & Hugenholtz, 1991; Torino *et al.*, 2005). Citric acid cannot be detected in grapefruit juice after fermentation in the case of *Lactobacillus* and mixed cultures of LAB and bifidobacteria, while amount of citric acid

concentration lost in the case of *B. bifidum* B7.5. Our results are in agreement with data reported by Mousavie *et al.* (2010). The initial concentration of citric acid in pomegranate juice was decreased significantly during the fermentation by investigated lactic acid bacteria (*L. plantarum, L. delbruekii, L. acidophilus* and *L. paracasei*) (Mousavi *et al.*, 2010).

**Table 4.8 Change of organic acids content during grapefruit juice fermentation**

| Strain | Lactic acid | Acetic acid | Citric acid | Oxalic acid |
|---|---|---|---|---|
| | Concentration (mM) | | | |
| *L. plantarum* 01 | 186 ± 3.5 | 1074 ± 28 | - | 10.7 ± 0.32 |
| *L. rhamnosus* B01725 | 172 ± 5.2 | 645 ± 30 | - | 9.8 ± 0.76 |
| *L. fermentum* D13 | 161 ± 6.4 | 539 ± 51 | - | 7.1 ± 0.21 |
| *B.bifidum* B7.5 | 9.6 ± 0.82 | 8.9 ± 1.6 | 77.7 ± 3.4 | 3.8 ± 0.11 |
| *L. plantarum* 01 + *B. bifidum* B7.5 | 190 ± 2.3 | 682 ± 9.5 | - | 9.3 ± 0.26 |
| *L. rhamnosus* B01725 + *B. bifidum* B7.5 | 161 ± 2.3 | 551 ± 12 | - | 9.4 ± 0.45 |
| *L. fermentum* D13+ *B.bifidum* B7.5 | 165 ± 0.2 | 670 ± 19 | - | 3.8 ± 0.15 |

In the case of *B. bifidum* B7.5 strain, the same amount of lactic acid and acetic acid were formed. The concentration of lactic acid and acetic acid in grapefruit juice were 9.6 mM and 8.9 mM, respectively after 24 hours of fermentation. While in the other studies, the significant higher of lactic acid was produced in carrot juice fermented by *Bifidobacterium* including *B. lactis* Bb-12, *B. bifidum* B3.2, *B. bifidum* B7.1 (Kun *et al.*, 2008), in fermentation of the apricot juice with *B. lactis* Bb-12 and *B. longum* Bb-46 (Bujna *et al.*, 2018). The initial concentration of oxalic acid in grapefruit juice was 2.3 mM. During fermentation process, concentrations of oxalic acid were increased in all cases of probiotic bacteria, they ranged from 3.8 to 10.7 mM. In the study on production of organic acids in different media, Zalán *et al.* (2009) concluded that the production of organic acids in lactic fermentation could be depended on the media and strains.

### 4.3.2. Changes of antioxidant capacity and total polyphenol content

Grapefruit juice is a rich source of bioactive compounds (Vanamala *et al.*, 2006; Zhang, 2007). Lactic fermentation of fruit juice provides "functional food", because it contains bioactive compounds such as fiber, oligosaccharides, and bacteria that promote the equilibrium of intestinal microflora (Jankovic *et al.*, 2010; Perricone *et al.*, 2015). Particularly, fruit juices do not contain any dairy allergens (e.g., lactose) that might be very suitable for who is lactose intolerance

(Luckow & Delahunty, 2004). Thus, the potential beverage produced by lactic acid fermentation of grapefruit juice would exert greater health effects in humans. Evaluation of the changes in total polyphenol content (TPC) and antioxidant capacity of grapefruit juice during fermentation and throughout the storage time were obtained in our study. Therefore, the combination of fruit juice with probiotic bacteria will provide a potential health benefiting beverage.

During the fermentation, the TPC of fermented grapefruit juice decreased in all cases. The highest TPC was observed in juice fermented by *B. bifidum* B7.5 strain with the TPC content of 1003 μg/mL garlic acid equivalent (**Table 4.9**). Fermentation with *B. bifidum* B7.5 strain reduced TPC from 1036 μg/mL at the beginning of experiment to 1003 μg/mL, representing a decrease of approximately 3.2% after 24 h of grapefruit juice fermentation. During storage, the TPC decreased further to 850 μg/mL garlic acid equivalent. Different TPC values were determined in the samples fermented by mono and mixed cultures. In the case of *L. fermentum* D13 strain, significantly the highest TPC (908 μg/mL garlic acid equivalent, $p < 0.05$) was measured after 12 weeks of storage, meaning about 12% reduction. For other mono and mixed cultures, the TPC was not significantly different at the end of fermentation or of storage period.

The antioxidant capacity of grapefruit juice did not change significantly in the case of *B. bifidum* B7.5 strain, decreasing slightly from 8.57 to 8.23 mM (**Table 4.10**). Significant decrease of antioxidant capacity was observed in all other cases, especially for fermentation of grapefruit juice by *L. rhamnosus* B01725 strain, where the lowest antioxidant capacity of 7.72 mM was measured. Due to anaerobic fermentation, fermented grapefruit juice is not exposed to oxygen, leading to minimal decrease in TPC as well as antioxidant capacity values. The antioxidant activity in the grapefruit juice fermented with probiotic bacteria decreased significantly during storage at 4 °C. About 44% of the antioxidant capacity was lost at week 12 of storage in the case of *B. bifidum* B7.5 strain and in combinations of *B. bifidum B7.5* strain with lactobacilli. Recent reports have studied the effect of lactic acid fermentation on antioxidant properties of food products. Tien *et al.* (2005) studied the lactic acid fermentation of sugar apple puree by *L. delbrueckii* subsp. *lactis* ATCC7830, *L. paracasei* subsp. *paracasei* ATCC 25598, and *L.casei* subsp. *casei* ATCC 393. The decrease in DPPH scavenging effects was observed in all cases of mono and mixed cultures for fermenting sugar apple puree and fresh apple sugar juice (Tien *et al.,* 2005). An increase in antioxidant properties was obtained in carrot juice fermentation by *L. bulgaricus* and *L. rhamnosus* (Nazzaro *et al.,* 2008). The increase in antioxidant capacity associated with *B. longum*, *L. casei*, and *L. plantarum* was found during 72 h fermentation of noni juice (Wang *et al.*, 2009). Based on the findings of the effect of lactic acid fermentation on antioxidant property, it may be concluded

that the antioxidant activity varied with starter microorganisms and may not be affected synergistically (Bujna *et al.*, 2018).

**Table 4.9 Changes of TPC values of fermented grapefruit juice during fermentation and storage by mono and mixed cultures**

| | *L. plantarum* 01 | *L. rhamnosus* B01725 | *L. fermentum* D13 | *B. bifidum* B7.5 | *L. plantarum* 01 + *B. bifidum* B7.5 | *L. rhamnosus* B01725 + *B. bifidum* B7.5 | *L. fermentum* D13 + *B. bifidum* B7.5 |
|---|---|---|---|---|---|---|---|
| 0h | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ | $1036^{Aa} \pm 16$ |
| 6h | $997^{ABb} \pm 16$ | $1006^{ABab} \pm 12$ | $991^{Bb} \pm 34$ | $1029^{Aab} \pm 20$ | $1008^{ABab} \pm 14$ | $989^{Bab} \pm 14$ | $995^{ABab} \pm 10$ |
| 12h | $983^{Ab} \pm 17$ | $996^{Abc} \pm 5$ | $989^{Abc} \pm 38$ | $1009^{Ab} \pm 17$ | $991^{Aab} \pm 9$ | $984^{Aabc} \pm 12$ | $976^{Ab} \pm 17$ |
| 18h | $991^{ABb} \pm 28$ | $973^{Bcd} \pm 21$ | $986^{ABbc} \pm 27$ | $1013^{Aab} \pm 8$ | $981^{ABbc} \pm 25$ | $989^{ABab} \pm 14$ | $986^{ABab} \pm 12$ |
| 24h | $984^{ABb} \pm 12$ | $975^{Bbcd} \pm 6$ | $994^{ABb} \pm 23$ | $1003^{Ab} \pm 14$ | $975^{Bbc} \pm 15$ | $980^{ABabc} \pm 12$ | $980^{ABb} \pm 19$ |
| 2w | $932^{Bc} \pm 17$ | $986^{Abcd} \pm 18$ | $1007^{Aab} \pm 5$ | $947^{Bc} \pm 11$ | $937^{Bc} \pm 15$ | $944^{Bbcd} \pm 10$ | $952^{Bbc} \pm 2$ |
| 4w | $939^{BCc} \pm 6$ | $959^{ABde} \pm 7$ | $978^{Abc} \pm 16$ | $871^{Dde} \pm 18$ | $935^{BCc} \pm 10$ | $921^{Ccde} \pm 17$ | $948^{Bbc} \pm 16$ |
| 6w | $888^{CDde} \pm 3$ | $934^{Bef} \pm 5$ | $972^{Abc} \pm 12$ | $848^{Ee} \pm 8$ | $872^{CDEd} \pm 12$ | $867^{DEef} \pm 26$ | $903^{BCcd} \pm 40$ |
| 8w | $918^{ABcd} \pm 4$ | $908^{ABfg} \pm 24$ | $948^{Abcd} \pm 5$ | $875^{BCd} \pm 13$ | $839^{Cde} \pm 34$ | $908^{ABde} \pm 82$ | $877^{BCd} \pm 29$ |
| 10w | $876^{ABe} \pm 9$ | $886^{Ag} \pm 12$ | $925^{Ad} \pm 15$ | $875^{ABd} \pm 13$ | $814^{Ce} \pm 44$ | $802^{Cfg} \pm 52$ | $820^{BCe} \pm 46$ |
| 12w | $820^{Bf} \pm 33$ | $839^{Bh} \pm 31$ | $908^{Ad} \pm 8$ | $850^{ABde} \pm 6$ | $790^{Be} \pm 46$ | $786^{Bg} \pm 37$ | $805^{Be} \pm 58$ |

Different lowercase superscript letters in the same column (different sampling time) and different uppercase superscript letters in the same row (different inoculation starter) show significant difference according to the Tukey's test ($p<0.05$)

**Table 4.10 Changes of antioxidant capacity of fermented grapefruit juice during fermentation and storage by mono and mixed cultures**

| | *L. plantarum* 01 | *L. rhamnosus* B01725 | *L. fermentum* D13 | *B.bifidum* B7.5 | *L. plantarum* 01 + *B. bifidum* B7.5 | *L. rhamnosus* B01725 + *B.bifidum* B7.5 | *L. fermentum* D13 + *B.bifidum* B7.5 |
|---|---|---|---|---|---|---|---|
| 0h | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ | $8.57^{Aa} \pm 0.09$ |
| 6h | $8.22^{Aab} \pm 0.38$ | $8.27^{Aab} \pm 0.2$ | $8.14^{Ab} \pm 0.29$ | $7.94^{Ab} \pm 0.14$ | $8.14^{Aab} \pm 0.13$ | $8.1^{Ab} \pm 0.14$ | $8.01^{Ab} \pm 0.21$ |
| 12h | $8.21^{Aab} \pm 0.15$ | $8.06^{Abc} \pm 0.19$ | $8.15^{Ab} \pm 0.41$ | $8.05^{Ab} \pm 0.2$ | $8.06^{Ab} \pm 0.1$ | $8.16^{Ab} \pm 0.32$ | $7.89^{Ab} \pm 0.34$ |
| 18h | $8.16^{Ab} \pm 013$ | $7.97^{Abc} \pm 0.12$ | $1.98^{Ab} \pm 0.15$ | $8.07^{Ab} \pm 0.15$ | $7.96^{Ab} \pm 0.31$ | $7.97^{Ab} \pm 0.15$ | $8.05^{Ab} \pm 0.23$ |
| 24h | $7.9^{ABb} \pm 0.27$ | $7.72^{Ac} \pm 0.25$ | $8.05^{ABb} \pm 0.25$ | $8.23^{Aab} \pm 0.18$ | $7.94^{ABb} \pm 0.19$ | $8.08^{ABb} \pm 0.11$ | $8.05^{ABb} \pm 0.29$ |
| 2w | $6.22^{Dc} \pm 0.07$ | $6.43^{CDd} \pm 0.05$ | $6.6^{BCc} \pm 0.18$ | $6.54^{BCDc} \pm 0.29$ | $6.29^{CDc} \pm 0.31$ | $6.86^{Bc} \pm 0.13$ | $7.24^{Ac} \pm 0.07$ |
| 4w | $5.83^{Cd} \pm 0.14$ | $5.61^{CDe} \pm 0.39$ | $6.55^{ABc} \pm 0.03$ | $5.34^{Dd} \pm 0.47$ | $6.41^{Bc} \pm 0.16$ | $6.41^{Bd} \pm 0.15$ | $6.99^{Ac} \pm 0.2$ |
| 6w | $5.04^{Be} \pm 0.27$ | $4.97^{Bf} \pm 0.29$ | $5.97^{Ad} \pm 0.17$ | $4.56^{Be} \pm 0.04$ | $6.27^{Ac} \pm 0.63$ | $5.71^{Ae} \pm 0.25$ | $6.16^{Ad} \pm 0.34$ |
| 8w | $4.76^{DEef} \pm 0.05$ | $4.78^{DEf} \pm 0.21$ | $5.5^{Be} \pm 0.02$ | $4.69^{Ee} \pm 0.21$ | $5.21^{Cd} \pm 0.09$ | $4.94^{Df} \pm 0.11$ | $6.24^{Ad} \pm 0.08$ |
| 10w | $4.5^{Cfg} \pm 0.15$ | $4.86^{Bf} \pm 0.12$ | $4.72^{Bf} \pm 0.05$ | $4.73^{Be} \pm 0.07$ | $4.88^{BCd} \pm 0.15$ | $4.71^{Bf} \pm 0.14$ | $5.4^{Ae} \pm 0.12$ |
| 12w | $4.29^{Cg} \pm 0.06$ | $4.57^{Bf} \pm 0.17$ | $4.46^{ABf} \pm 0.09$ | $4.84^{Ae} \pm 0.15$ | $4.85^{Ad} \pm 0.19$ | $4.83^{Af} \pm 0.08$ | $4.81^{Af} \pm 0.02$ |

Different lowercase superscript letters in the same column (different sampling time) and different uppercase superscript letters in the same row (different inoculation starter) show significant difference according to the Tukey's test ($p<0.05$)

### 4.3.3. Changes in naringin concentrations

The grapefruit juice was fermented with mono and mixed probiotic cultures that exhibited high naringinase production. The results are summarized in **Figure 4.14**.

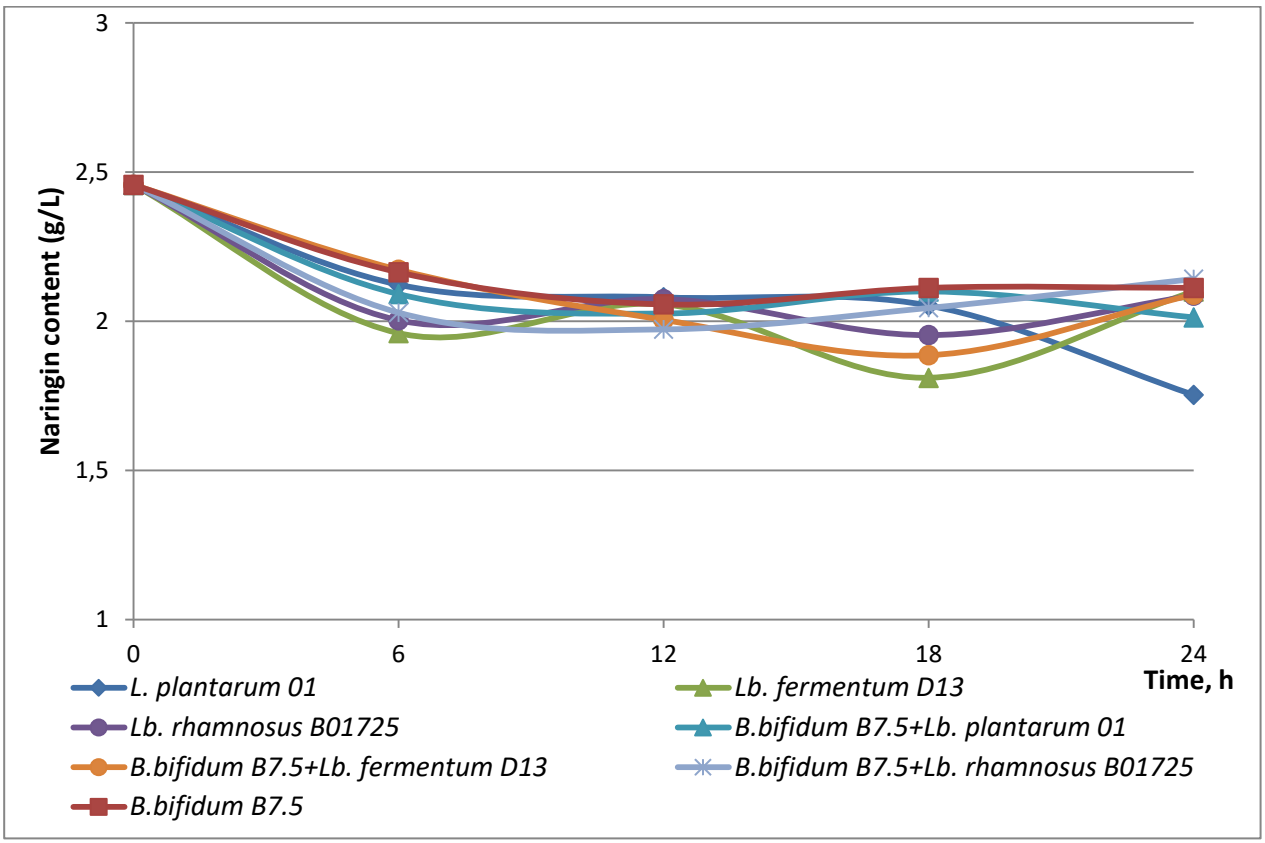


**Figure 4.14 Changes in naringin concentrations during fermentation by mono and mixed cultures of probiotic starters**

The initial naringin content in the grapefruit juice was about 2.5 g/L. Among all cases, the maximum decrease of naringin (about 28%) was obtained after 24 h of fermentation by using mono starter *L. plantarum* 01 strain (**Fig. 4.14**). This result might be explained by the origin of the *L. plantarum* strain, as it was isolated from plant. In view of the frequent occurrence of lactobacilli on decaying plant material and fermented vegetable substrates, one could anticipate that their genomes carry one or more genes encoding enzymes capable of utilizing rhamnosilated compounds (Beekwilder *et al.*, 2009).

The application of naringinase for debitterization of orange juice was report by Zhu *et al.* (2017). The purified naringinase from *Aspergillus oryzae* 1125 with 8 U/mL was used to decrease the naringin concentration below 30 μg/mL and prove that the bitterness can be efficiently decreased by naringinase below the threshold of taste (Zhu *et al.*, 2017a). Similar result was presented in debittering citrus juice using naringinase from *Aspergillus niger*. It was observed that

when the naringinase was maintained at or above 0.222 U/mL, the naringin concentration decreased to below 15 μg/mL, which is lower than the taste threshold of naringin in juice (about 20 ppm) (Ni *et al.*, 2012a). Another study of Zhu *et al.* (2017) showed that the isolated naringinase was capable of hydrolyzing naringin in citrus juice below 30 μg/mL with the maintaining of naringinase levels at or higher than 4 U/mL (Zhu *et al.*, 2017b). In another study on debittering of grapefruit juice with purified naringinase from *Cryptococcus albidus*, the concentration of naringin was reduced by 84% at 40 °C and 100% at 60 °C after 60 min of incubation (Borzova *et al.*, 2018).

The biotechnological potential of naringinase producing yeast, *Clavispora lusitaniae,* has been exploited for the processing of "Kinnow and Lemon" a low-alcoholic, naturally carbonated, debittered, fermented beverage (Pandove *et al.*, 2017). Three months of storage at refrigerated temperature revealed that the naringin content in fermented Kinnow:Lemon beverage decreased from 443.20 ppm down to 176.4 ppm.

In our experiments we could not achieve as efficient debittering of grapefruit juice by naringinase produced by our lactic acid bacteria as in other studies in the literature with purified enzyme supplementation. However, this may be the first time that reduction of naringin in grapefruit juice is reported by living probiotic bacteria, when fermenting of grapefruit juice is combined with debittering.

## 5. NOVEL CONTRIBUTIONS

1. Sixteen strains of genera *Lactobacillus* and twenty strains of genera *Bifidobacterium* ssp were screened for naringinase activity. These strains secreted extracellular naringinase in presence of naringin as producer after 24 hours of fermentation and *L. fermentum* D13 strain was selected for further studies.

2. Different strategies were applied to investigate effects of medium compositions and physical parameters on production of naringinase. Sucrose was the best carbon source for enzyme production. The optimum amount of inoculum, pH, naringin concentration and sucrose concentration were determined to be 4% (v/v), pH 6.1, 0.1% (w/v) and 6.0 g/L, respectively. The presence of 5 mM $Cu^{2+}$ in the MRS fermentation medium stimulated naringinase synthesis. The maximum naringinase activity was 5 IU/mL under optimal conditions.

3. The maximum activity of crude naringinase from *L. fermentum* D13 strain was achieved at pH 4.5 and temperature 45 °C. The presence of $Cu^{2+}$ has a significant positive effect on the enzymatic activity.

4. Strategy for simultaneous debittering and production of probiotic grapefruit juice was designed and carried out. Four strains *L. plantarum* 01, *L. rhamnosus* B01725, *L. fermentum* D13 and *B. bifidum* B7.5 were selected for monocultural and mixed cultural fermentation of grapefruit juice. Lactic acid bacteria were able to grow well on grapefruit juice without any nutrition supplements. After 24 h of fermentation, the microbial population achieved about $10^9$ cfu/mL. The maximum decrease in naringin content (about 28%) was obtained after 24 h of monocultural fermentation by *L. plantarum* 01 strain.

## 6. SUMMARY

Lactic acid bacteria, including 16 strains of *Lactobacillus* and 20 strains of *Bifidobacterium* from the fermentation lab of Research Centre for Bioengineering and Process Engineering, Faculty of Food Science, Szent István University, Hungary, were used for screening naringinase production. The lactobacilli were grown aerobically at 37 °C in MRS broth without meat extract and lower glucose concentration (3 g/L), while the bifidobacteria were incubated anaerobically in TPY broth. Both media were modified with 0.05% naringin, and the Davis method was used to evaluate the naringinase activity. *L. fermentum* D13 strain showed the best production of naringinase after culturing at 37 °C for 24 h, thus it was selected for further studies.

*L. fermentum* D13 strain was used for naringinase production and to investigate the factors affecting production of naringinase, such as ratio of inoculum bacteria, pH, naringin concentration, different carbohydrate sources and metal ions. Different ratios of bacteria (2%, 4%, 6% and 8%) were inoculated into the medium for fermentation with the cell count of $10^9$ cfu/mL. The ratio of inoculated bacteria of 4% (v/v) and initial pH 6.0 showed the highest yield of naringinase. Carbohydrate sources affected naringinase production by *L. fermentum* D13 strain. Sucrose was the best carbon source resulting in the highest naringinase activity of 3.95 IU/mL. The maximal enzyme production was obtained at a concentration of sucrose of 5 g/L, though molasses, glucose, and galactose also supported naringinase production. Metal ions play important role in hydrolysis reactions done by enzymes. While $Cu^{2+}$ and $Zn^{2+}$ stimulated naringinase synthesis, $Fe^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$ inhibited naringinase activity at concentration of 5 mM. The maximum naringinase activity was achieved, when 5 mM $Cu^{2+}$ was added to the fermentation medium.

Factors pH, naringin and sucrose contents on naringinase production were optimized by response surface methodology. The maximum naringinase activity, 5 IU/mL, was observed at pH 6.1, naringin content 0.1% (w/v) and sucrose concentration 6.0 g/L.

Crude enzyme naringinase was lyophilized at 0.25 MPa, 17 °C from the supernatant of fermentation broth. The optimum pH and temperature were pH 4.5 and 40 °C, respectively, for the hydrolysis of naringin by crude naringinase. It is interesting that metal $Cu^{2+}$ also had positive effect on the enzymatic activity. Relative enzyme activity was significantly higher (about 60%) than without adding metal ion.

Probiotic bacteria producing naringinase were applied for lactic acid fermentation of grape fruit juice and reducing bitterness at the same time. The pH of the commercial grape fruit juice was adjusted to pH 6.3 before inoculated with mono- or mixed bacterial cultures. Four mono

cultures of lactobacilli and bifidobacteria, including *L. plantarum* 01, *L. rhamnosus* B01725, *L. fermentum* D13, and *B. bifidum* B7.5 strain and three combinations of *Lactobacillus* and *Bifidobacterium* strains were used for lactic acid fermentation of grapefruit juice, and reduction of bitterness was followed during fermentation. Antioxidant capacity, TPC, and the microbial population of fermented grapefruit juice were analyzed during fermentation and 12 weeks of storage at 4 °C. In all cases, lactic acid bacteria showed to be capable of growing well on sterilized grapefruit juice without nutrition supplementation. After 24 h of fermentation, the microbial population achieved about $10^9$ cfu/mL, so the grapefruit juice could be a potential growth medium for producing functional beverage. The maximum decrease of naringin (about 28%) was obtained after 24 h of fermentation by monoculture *L. plantarum* 01 strain as starter. The antioxidant capacity and the TPC of the fermented grapefruit juice slightly decreased during fermentation. These values continued to decrease during the storage period. At the 12th week, reduction of antioxidant capacity and TPC were about 43-50% and 12-24%, respectively, compared to the initial values. After 12 weeks of storage at 4 °C, the surviving cell counts in the fermented grapefruit juice still remained $10^8$–$10^9$ cfu/mL in most cases, with the exception of juice fermented by monoculture *L. fermentum* D13 strain, where the viable cell count was $9\times10^7$ cfu/mL, 5-12 times lower.

Overall, in our experiments we could not achieve as efficient debittering of grapefruit juice by naringinase produced by our lactic acid bacteria as in other studies in the literature with purified enzyme supplementation. However, this may be the first time that reduction of naringin in grapefruit juice is reported by living probiotic bacteria, when fermenting of grapefruit juice is combined with debittering.

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