FERMENTATIVE PRODUCTION OF DEBITTERED KINNOW BEVERAGE USING α-L-RHAMNOSIDASE PRODUCING YEAST

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
in
MICROBIOLOGY
(Minor Subject: Biochemistry)

By

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(L-2011-BS-67-D)

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2015

CERTIFICATE - I

This is to certify that the dissertation entitled, **"FERMENTATIVE PRODUCTION OF DEBITTERED KINNOW BEVERAGE USING α-L-RHAMNOSIDASE PRODUCING YEAST"** submitted for the degree of **Doctor of philosophy**, in the subject of **Microbiology (Minor subject: Biochemistry)** of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Pratiksha Singh (Admission No. L-2011-BS-67-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that the dissertation entitled, **"FERMENTATIVE PRODUCTION OF DEBITTERED KINNOW BEVERAGE USING α-L-RHAMNOSIDASE PRODUCING YEAST"** submitted by **Pratiksha Singh (Admission No. L-2011-BS-67-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of Ph.D, in the subject of **Microbiology (Minor subject: Biochemistry)** has been approved by the Student's Advisory Committee after an oral examination on the same.

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ABSTRACT

The production of α-L- rhamnosidase from *Clavispora lusitaniae* (KF633446) has been characterized and evaluated for its effectiveness in debittering citrus juice. *Clavispora lusitaniae* produced 0.106 IU mL^{-1} enzyme activity in minimal media using rhamnose as the main carbon source. The enzyme has been purified to homogeneity by sulfate fractionation and DEAE- sephadex column chromatography, and measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be around 85 kDa. The enzyme has been purified to 10.1- fold with 46.98% recovery, 24.9 IU activity and 2.7 IU mg^{-1} specific activity. The pH and temperature optima of enzyme are 4 and 50 °C, respectively. The Michaelis- Menton constants for the hydrolysis of p-nitrophenyl α-L-rhamnopyranoside are 0.18 mM and 25 IU mL^{-1}. The enzyme is thermostable up to 50 °C and operational stability in kinnow juice. Metal ion K^+ affected positively the activity and Ag^+ completely inhibited the activity. The lyophilized enzyme powder could retain its enzymatic activity at 4 °C for 3 months. The enzyme (0.8 IU mL^{-1}) in citrus juice could hydrolyze the naringin from 600 ppm to palatable bitterness (180.71 ppm). Oral acute toxicity study revealed the enzyme is nontoxic and safe for food use. The contents of arsenic (As), lead (Pb), cadmium (Cd) in the enzyme powder met the criteria for food use. The optimized parameters for debittering of kinnow juice are; enzyme activity (0.8 IU mL^{-1}), TSS (13 °B), temperature (30±5°C) and incubation time (4 h) and for kinnow beverage; yeast inoculum concentration (0.75% v/v), TSS (13 °B), temperature (30±5 °C) and incubation time (48 h). These characteristics suggest that the α-L-rhamnosidase from *Clavispora lusitaniae* holds potential for debittering the citrus juice, and the bioprocess consisting of production, salt precipitation, dialysis, ion exchange chromatography and lyophilization, a promising means to prepare the purified α-L-rhamnosidase for commercially and setting up a strong base to enzymatically debittered citrus juice.

Keywords: Naringin, Prunin, α-L-rhamnosidase, *C. lusitaniae*, Purification, Beverage

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Signature of Major Advisor **Signature of the student**

ਖੋਜ ਗ੍ਰੰਥ ਦਾ ਸਿਰਲੇਖ	:	α-ਐਲ-ਰਹੈਮਨੋਸੀਡੇਜ਼ ਦਾ ਉਤਪਾਦਨ ਕਰਨ ਵਾਲੇ ਖਮੀਰ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਕਿੰਨੂ ਦਾ ਕੁੜਤਣ ਰਹਿਤ ਜੂਸ ਤਿਆਰ ਕਰਨਾ
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ਸਾਰ

ਕੁੜਤਣ ਭਰਪੂਰ ਨੈਰਿੰਨਜਿਨ ਤੋਂ ਕੁੜਤਣ ਰਹਿਤ ਪਰੂਨਿਨ ਅਤੇ ਰਹੈਮਨੋਜ਼ ਦੀ ਹਾਈਡ੍ਰੋਲਾਈਜ਼ਿੰਗ ਦੁਆਰਾ ਕਿੰਨੂ ਦੇ ਜੂਸ ਦੀ ਕੁੜਤਣ ਦੂਰ ਕਰਨ ਲਈ α-ਐਲ-ਰਹੈਮਨੋਸੀਡੇਜ਼ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਜਾਂਦੀ ਹੈ। ਸਿਰਫ ਚਾਰ ਨਿਖੇੜਕ (84, B82, 86 and S82) α-ਐਲ-ਰਹੈਮਨੋਸੀਡੇਜ਼ ਇੰਜ਼ਾਇਮ (i.e. 0.058, 0.046, 0.033 ਅਤੇ 0.029 IU mL^{-1}) ਬਣਾਉਂਦੇ ਹਨ। BIOLOG ਫਿਨੋਟਾਈਪ ਮਾਈਕ੍ਰੋਐਸੇ ਪਲੇਟਾਂ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਕਾਰਬਨ ਸਬਸਟ੍ਰੇਟ ਉਪਯੋਗਤਾ ਪ੍ਰੋਫਾਈਲਿੰਗ ਦੇ ਅਧਾਰਤ ਤੇ ਜੀਵ-ਰਸਾਇਣਕ ਗੁਣਾਂ ਦਾ ਵਿਸ਼ਲੇਸ਼ਣ ਕੀਤਾ ਗਿਆ। ਰਾਈਬੋਸੋਮਲ DND ਦੇ ITS ਖੇਤਰ ਦੇ ਆਣਵਿਕ ਵਿਸ਼ਲੇਸ਼ਣ ਦੇ ਅਧਾਰ ਤੇ, ਖਮੀਰ ਨਿਖੇੜਕਾਂ ਦੀ ਪਹਿਚਾਣ ਵਜੋਂ *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ* (84), *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ* (82), *ਕੈਂਡੀਡਾ* ਸਪੀਸੀਜ਼ (86) ਅਤੇ *ਕੈਂਡੀਡਾ ਹਾਈਡ੍ਰਾਬੈਡੈਂਸਿਸ* (S82) ਕੀਤੀ ਗਈ। ਰੈਪੀਟੇਟਿਵ ਡੀ.ਐਨ.ਏ. ਅਨੁਕ੍ਰਮ (ERIC, REP ਅਤੇ BOX) ਅਧਾਰਿਤ ਡੀ.ਐਨ.ਏ. ਫਿੰਗਰ ਪ੍ਰੀਟਿੰਗ ਦੀ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਸੰਪੂਰਨ ਜੀਨੋਮ ਮੁਲਾਂਕਣ ਰਾਹੀਂ ਨਿਖੇੜਕਾਂ ਵਿੱਚ ਅੰਤਰ-ਪ੍ਰਜਾਤੀ ਅਨੁਵਾਂਸ਼ਿਕੀ ਵਿਭਿੰਨਤਾ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਖਮੀਰ ਸਟ੍ਰੇਨ *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ* (84) ਨੇ ਰਹੈਮਨੋਜ਼ 0.6 w/v, ਖਮੀਰ ਅਰਕ 0.4% w/v, ਤਾਪਮਾਨ 35°C ਅਤੇ ਪੀ.ਐਚ. 4 ਉਪਰ ਨੈਰਿੰਨਜਿਨ ਯੁਕਤ ਸਪਲੀਮੈਂਟਿਡ ਮੀਡੀਅਮ ਵਿੱਚ ਸਭ ਤੋਂ ਵਧੇਰੇ ਰਹੈਮਨੋਸਾਈਡੇਜ਼ ਗਤੀਵਿਧੀ (0.106 IU mL^{-1}) ਵਿਖਾਈ। ਬਹੁ ਵਭਿੰਨਰੂਪੀ ਰਿਸਪੋਂਸ ਸਰਫੇਸ ਮੈਥੇਡੋਲੋਜੀ ਦੁਆਰਾ ਇੰਜ਼ਾਈਮ ਗਤੀਵਿਧੀ ਅਤੇ ਇਸ਼ਟਤਮ ਇੰਜ਼ਾਈਮ ਉਤਪਾਦਨ ਉਪਰ ਵੱਖੋ-ਵੱਖਰੇ ਮਾਪਦੰਡਾਂ ਦਾ ਪ੍ਰਭਾਵ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਫਿਟ ਆਫ ਮਾਡਲ (R^2= 0.409479) ਅਰਥਪੂਰਨ ਪਾਇਆ ਗਿਆ। ਕ੍ਰੋਮੈਟੋਗ੍ਰਾਫਿਕ ਅਤੇ ਇਲੈਕਟ੍ਰੋਫੋਰੇਟਿਕ ਵਿਧੀਆਂ ਵਰਤੋਂ ਕਰਕੇ ਸ਼ੁਧੀਕ੍ਰਿਤ ਕੀਤੇ ਗਏ ਇੰਜ਼ਾਇਮਾਂ ਦੀ ਹੋਮੋਜੀਨਾਇਟੀ SDS-PAGE ਦੁਆਰਾ 85 kDa ਆਂਕੀ ਗਈ। ਪੀ-ਨਾਈਟ੍ਰੋਫਿਨਾਇਲ α-ਐਲ-ਰਹੈਮਨੋਪਾਇਰਾਨੋਸਾਈਡ ਇੰਜ਼ਾਈਮ ਲਈ Km ਅਤੇ Vmax ਦੀ ਮਿਕਦਾਰ ਕ੍ਰਮਵਾਰ 0.18 mM ਅਤੇ 25 IU mL^{-1} ਦਰਜ ਕੀਤੀ ਗਈ। 4 ਪੀ.ਐਚ. ਅਤੇ 50 °C ਤਾਪਮਾਨ ਇੰਜ਼ਾਈਮਾਂ ਲਈ ਸਹੀ ਪਾਇਆ ਗਿਆ। ਸ਼ੁਧੀਕ੍ਰਿਤ ਲਾਈਓਫੀਲਾਇਜ਼ਡ ਇੰਜ਼ਾਈਮ ਵਿਸ਼ੈਲ ਰਹਿਤ ਅਤੇ ਭੋਜਨ ਪਦਾਰਥ ਵਿੱਚ ਖਪਤ ਲਈ ਸੁਰੱਖਿਅਤ ਸਨ ਅਤੇ ਕਿੰਨੂ ਦੇ ਪਤਲੇ ਜੂਸ (13 °B) ਵਿੱਚ ਦੀ ਖਟਾਸ ਘਟਾਉਣ ਲਈ 0.8 IU mL^{-1} ਇੰਜ਼ਾਈਮ ਗਤੀਵਿਧੀ ਕਾਫੀ ਸੀ। ਇਸ਼ਟਤਮ ਹਲਾਤਾਂ (ਖਮੀਰ ਇਨੋਕੁਲਮ ਘਣਤਾ 0.75% v/v, ਟੀ.ਐਸ.ਐਸ. 13°B, ਤਾਪਮਾਨ 30°C ਅਤੇ ਇਨਕੁਬੇਸ਼ਨ ਸਮਾਂ 48 ਘੰਟੇ) ਉਪਰ *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ* (84) ਦੁਆਰਾ ਉਤਪਨ ਇੰਜ਼ਾਈਮ (0.8 IU mL^{-1}) ਦੀ ਵਰਤੋਂ ਨਾਲ ਕਿੰਨੂ ਦਾ ਕੁੜਤਣ ਰਹਿਤ ਜੂਸ ਤਿਆਰ ਕੀਤਾ ਗਿਆ। ਭੰਡਾਰਨ ਦੌਰਾਨ ਜੂਸ ਦੇ ਭੌਤਿਕ ਰਸਾਇਣਕ ਮਾਪਦੰਡ ਉਪਰ ਕੋਈ ਅਰਥਪੂਰਨ ਬਦਲਾਅ ਨਹੀਂ ਅਤੇ ਅਤੇ ਇਸਦੀ ਮਿਆਦ ਤਿੰਨ ਮਹੀਨੇ ਆਂਕੀ ਗਈ। ਇਸ ਲਈ *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ* (84) ਤੋਂ ਪ੍ਰਾਪਤ α-ਐਲ-ਰਹੈਮਨੋਸੀਡੇਜ਼ ਇੰਜ਼ਾਇਮ ਰਾਹੀਂ ਜੂਸ ਵਿੱਚਲੀ ਕੁੜਤਣ ਦੀ ਸਮੱਸਿਆ ਨੂੰ ਦਾ ਹੱਲ ਲੱਭਿਆ ਜਾ ਸਕਦਾ ਹੈ।

ਮੁੱਖ ਸ਼ਬਦ: ਨੈਰਿੰਨਜਿਨ, α-ਐਲ-ਰਹੈਮਨੋਸੀਡੇਜ਼, *ਕਲੈਵੀਸਪੋਰਾ ਲੁਸੀਟੇਨੀਆਇ,* ਸ਼ੁਧੀਕਰਨ, ਪੇਅ ਪਦਾਰਥ

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ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖ਼ਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖ਼ਰ**

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Chapter- I

INTRODUCTION

India is the second largest producer of fruits, with a production of 44.04 million tonnes of fruits from an area of 3.72 million hectares and holds third rank in respect of production of citrus fruits in the world. Kinnow, a hybrid of *Citrus nobilis* and *Citrus delicosa* is a prevalent citrus fruit in Punjab covering an area of 46,000 hectares with the production of 9.88 lakh tones NHM (2014).

Kinnow mandarin juice has high therapeutic value as antispasmodic, sedative, cytophylactic, digestive, anti carcinogenic, anti inflammatory and anti allergic. The health benefits of citrus fruit juices have been attributed due to the presence of bioactive and antioxidant compounds such as ascorbic acid (53 mg), flavonoids, limonoids, coumarins and essential vitamins: folates (30 µg), niacin (0.282 mg), panthothenic acid (0.25 mg), pyridoxine (0.060 mg), riboflavin (0.040 mg), thiamine (0.1 mg), vitamin A (225 IU), vitamin E (0.18 mg) per 100 g. Citrus fruits also contain minerals: calcium (40 mg), copper (39 mg), iron (0.1 mg), magnesium (10 mg), manganese (0.024 mg), zinc (0.8 mg) and β-carotenoides (71 µg) per 100 g (USDA National Nutrient Database). The nutritional and therapeutic value of kinnow provides ample scope for processing into a value added fermented product. Compared to fruit juices, the formulation of naturally carbonated beverage offers more variety of flavors, nutrients, long shelf life and other physiological benefits with a greater margin of safety in a drink with a lower inherent cost. Physiological benefits include retention of organoleptic properties, nutritional attributes, characteristics sensory properties, flavour, aroma, texture and long shelf life.

Kinnow juice processing has commercial restrictions due to bitter taste by chemical naringin (flavanoid) and limonin (limonoid). Naringin is the major component in citrus fruit which imparts bitter taste, a threshold of 20 mg kg^{-1} in water and detectable limit less than 1.5 mg kg^{-1} (Chen *et al* 2010). The presence of limonin and naringin in excess of 6 ppm and 600 ppm respectively has been established as an objectionable level of bitterness in processed citrus products such as juice, wine and vinegar (Guadagni *et al* 1973). Limonin is an intensely bitter compound highly oxygenated triterpenoid derivative comprising of a furan ring and an epoxide group. Intact fruit tissues do not contain limonin instead contain non-bitter precursor of limonin, limonoate A-ring lactone (LARL). LARL is found to be endogenously present in membranous sacs. During juice processing sac rupture, the LARL encounters the net acidic pH of the juice, which gradually catalyzes closure of the ring to form limonin.

Numerous techniques are used to reduce naringin: adsorptive debittering (Fayoux *et al* 2007), poly-styrene divinyl benzene styrene resin treatment and β-cyclodextrin treatment (Mongkolkul *et al* 2006). These techniques have limitations in altering nutrient composition

either through chemical reactions or removal of nutrients, flavor and color etc. In comparison, the reduction of naringin concentration by enzyme naringinase is one of the promising and economical debittering processes with the advantages of high efficiency, retention of nutrients, increasing acceptability by consumer and a convenient operation for removing the bitterness in large-scale commercial production (Yadav *et al* 2010).

The enzyme naringinase is composed of *α*-L-rhamnosidase (EC 3.2.1.40) and *β*-D-glucosidase (EC 3.2.1.21). α-L-Rhamnosidase cleaves terminal α-L-rhamnose specifically from a large number of natural products which include naringin, rutin, quercitrin, hesperidin, diosgene and ter-penyl glycosides (Feng *et al* 2005). Among flavonoids, naringin (4′,-5,7′-trihydroxyflavonone-7-rhamnoglucoside) can be hydrolyzed by α-L-rhamnosidase activity of naringinase to rhamnose and prunin (trihydroxyflavonone-7-glucoside) which can be further hydrolyzed into glucose and naringenin (4′-5,7′-trihydronyflavonone) by the β-D-glucosidase component of naringinase **(Fig. 1.1)**.

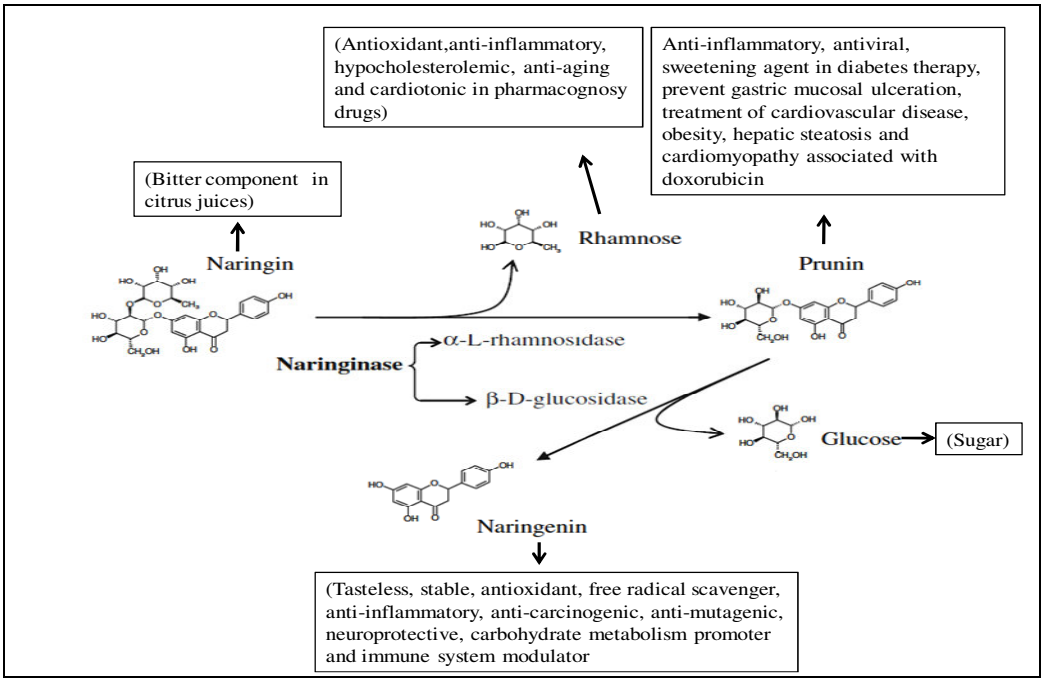


Fig. 1.1 (a) Hydrolysis of naringin into prunin and rhamnose by α-L-rhamnosidase
(b) Hydrolysis of prunin into naringenin and glucose by β-D-glucosidase

The α-L-Rhamnosidases (EC 3.2.1.40) has been used in several industrial and biotechnological processes. In food industry, rhamnosidases catalyze the hydrolysis of a wide spectrum of natural glycosides containing terminal L-rhamnose, enhancement of aroma such as the debittering of grapefruit juices by hydrolysis of naringin and derived beverages (Manzanares *et al* 2003, Puri and Kalra 2005), elimination of hesperidin crystals from orange

(Terada *et al* 1995) and a large number of natural products which include naringin, rutin, quercitrin, diosgene and ter-penyl glycosides (Feng *et al* 2005), the derhamnosylated product quercetin-3-glucoside from the flavonoid rutin increases the antioxidant activity of asparagus juice (Sun *et al* 2007). In chemical and pharmaceutical industries, this enzyme is used in the structural determination of polysaccharides, glycosides and glycolipids, metabolism of gellan (Hashimoto *et al* 1999), conversion of chloropolysporin B to chloropolysporin C, the derhamnosylation of many L-rhamnose containing steroids for example diosgene, desglucoruscin, ginsenosides Rg2, etc. whose derhamnosylated products have their clinical importance (Feng *et al* 2005) and production of prunin (Chandler and Nicol 1975) which possesses anti-inflammatory and antiviral activity against DNA/RNA viruses (Kaul 1985).

For optimization of any enzymatic processes, media components play an important role in enhancing the enzyme production. Rhamnosidase production mainly depends on the inducer, carbon and nitrogen source, reported inducers for naringinase production are rhamnose (Thammawat *et al* 2008), hesperidin (Fukumoto and Okada 1973), naringin (Bram and Solomons 1965, Puri *et al* 2008) and citrus peel powder (Puri *et al* 2011). Temperature is one of the most important variable affecting enzyme deactivation by weakening non-covalent interactions that stabilize the protein structure and leading to unfolding and subsequent changes that reduce the catalytic activity (Klibanov 1983), change in the pH value can also irreversibly change the protein structure by alteration of the charge of the amino acid responsible for maintenance of the secondary and tertiary structure (Bisswanger 1999). Extreme pH values lead similarly to chemical modification fully inactivating the enzyme. Enzyme concentration is also known to affect inactivation by modifying aggregation and subunit dissociation grade. So, the optimization of physical and nutritional conditions is very essential.

Optimizing the affecting parameters by statistical experimental designs can eliminate the limitations of a single factor optimization process collectively (Montogomery 2000). Response surface methodology (RSM) is a useful statistical technique for the investigation and optimization of complex processes. It uses quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation (Rastogi *et al* 2010). Central composite design (CCD) is a widely used response surface design when the experimental region is defined by the upper and lower limits of each factor and not extended beyond them (Neter *et al* 1996). A combination of factors generating a certain optimal response can be identified. Also, significant interactions between variables can be identified and quantified by this approach (Vishwanatha *et al* 2010).

The main sources of this enzyme are filamentous fungi (Monti *et al* 2004), have shown the induction of α-L-rhamnosidases production in the number of fungal strains such as *Acremonium persicinum*, *Circinella muscae*, *Emericella nidulans*, *Fusarium oxysporum*,

Mortierella alpina, *Penicillium oxalicum*, *Rhizopus arrhizus*, *Talaromyces flavus* and *Trichoderma harzianum*, using L-rhamnose, naringin, rutin, hesperidin as inducers. A number of *Aspergillus* species (*Aspergillus niger, A. terreus, A. nidulans* and *A. aculeatus*) have been reported for the production of α-L-rhamnosidases. Out of them only *Aspergillus niger* and *Penicillium decumbens* is the most commonly used for its production with potential value in oenology and their enzymatic activities have been well characterized (Gallego *et al* 1996, Orejas *et al* 1999, Manzanares *et al* 2003). Only two commercial preparations of α-L-rhamnosidases, naringinase and hesperidinase are available and both are from fungal sources. The biotechnological potentiality of these enzymes has led to the characterization of fourteen microbial α-L-rhamnosidase (GH78 family) encoding genes within the last decade. Of these only four were isolated from filamentous fungi: the genes encoding RhaA and RhaB of *Aspergillus aculeatus* (Manzanares *et al* 2001), AkRha78 of *Aspergillus kawachii* (Koseki *et al* 2008) and *Aspergillus nidulans* for AN10277 protein (Bauer *et al* 2006), here after named as rhaA.

Some bacterial strains producing α-L-rhamnosidases are thermophilic bacteria (Birgisson *et al* 2004), *Fusabacterium* (Park *et al* 2005), *Pseudoalteromonas species, Ralstonia pickettii* (Orrillo *et al* 2007), *Lactobacillus acidophilus* (Beekwilder *et al* 2009), *Pediococcus acidilactici* (Michlmayr *et al* 2011), *Clostridium stercorarium*, *Sphingomonas paucimobilis*, *Bacillus sp.* and *Corticium rolfsii*. The first bacterial α-L-rhamnosidase was purified from *Bacteroides* (Jang and Kim 1996). To date, the genes encoding α-L-rhamnosidases of *Clostridium stercorarium* (rhaA), *Bacillus sp.* GL1 (rhaA and rhaB) (Hashimoto 2003), *Sphingomonas paucimobilis* (rhaM) (Miyata *et al* 2005) and *Thermomicrobia* sp. (Birgisson *et al* 2004) have been cloned and characterized.

In few reports, low levels of activity were found in yeast belonging to genus *Saccharomyces, Hansenula, Debaryomyces, Candida, Aureobasidium pullulans* (Miklosy and Polos 1995; Rosi *et al* 1995; Mcmahon *et al* 1999; Yadav *et al* 2010) and *Pichia angusta* (Yanai and Sato 2000). *Clavispora lusitaniae* has been listed in FDA's approved microbial category and proven safe for food and medicinal use for eg. production of food grade enzymes (amylase, pectinase and protease), food additives and fermented beverage.

Many quality research works has been undertaken for debittering the kinnow juice below threshold level for acceptibility through physico-chemical/chemical/biotechnological approaches in order to utilize its immense potentiality in processed kinnow juice industry. Still there is no satisfactory method for removing or reducing bitterness without affecting the taste, aroma and colour of the juice. However, there is a lack of information on production, purification, characterization, preparation and evaluation of α-L-rhamnosidase from yeast in debittering of citrus juice.

Keeping all this in view the present research work has been carried under the following objectives:

1. Isolation and identification of α-L-rhamnosidase producing yeast strains.
2. Screening of media and juice components for optimization of α-L-rhamnosidase production from yeast.
3. Optimization of α-L-rhamnosidase production using Response Surface Methodology (RSM).
4. Purification, characterization, preparation and evaluation of α-L-rhamnosidase from yeast.
5. Upscale production of debittered kinnow juice and beverage and evaluating the beverage for organoleptic enhancement and prolonged shelf life.

Chapter- II

REVIEW OF LITERATURE

The literature related to present study on fermentative production of debittered kinnow beverage using α-L-rhamnosidases producing yeast has been reviewed under following headings:

2.1. Yeast

2.2. α-L-rhamnosidase

2.2.1 Sources of α-L-rhamnosidase

2.2.1.1 Plant α-L-rhamnosidase

2.2.1.2 Animal α-L-rhamnosidase

2.2.1.3 Microbial α-L-rhamnosidase

2.2.1.3.1 Yeast

2.2.1.3.2 Bacteria

2.2.1.3.3 Fungi

2.3. Kinnow production in India

2.4. Physicochemical characteristics of kinnow juice

2.4.1 Total sugars

2.4.2 Total soluble solids

2.4.3 Acidity

2.4.4 Ascorbic acid

2.5. Bitterness of citrus products

2.5.1 Flavonoid bitterness

2.5.2 Limonoid bitterness

2.6. Debittering of citrus fruit juices

2.6.1 Physicochemical approaches

2.6.1.1 Adsorptive debittering

2.6.1.2 Chemical methods

2.6.1.3 Debittering by passage through polystyrene- DVB resins

2.6.1.4 Debittering by β- cyclodextrin

2.6.2 Biotechnological approaches

2.7. Beverages

2.7.1 Ready to serve beverages

2.7.2 Blended beverages

2.7.3 Alcoholic beverages

2.7.4 Carbonated beverages

2.8. Microbial production of α-L-rhamnosidase enzyme

2.8.1 Effect of carbon sources on α-L-rhamnosidase production

2.8.2 Effect of nitrogen sources on α-L-rhamnosidase production

2.9. Optimization of α-L-rhamnosidase production using response surface methodology

2.10. Purification of α-L-rhamnosidase enzyme

2.11. Characterization of purified α-L-rhamnosidase enzyme

2.11.1 Effect of pH

2.11.2 Effect of temperature

2.11.3 Effect of enzyme and substrate concentration

2.11.4 Effect of metal ions and inhibitors

2.11.5 Molecular weight of rhamnosidase

2.11.6 Structure of rhamnoisidase

2.12. Molecular biology of rhamnosidase

2.13. Applications of α-L-rhamnosidase enzyme

2.13.1 Food industry

2.13.1.1 Debittering citrus fruit juices

2.13.1.2 Aroma enhancement

2.13.1.3 Gellan depolymerization

2.13.1.4 Tomato pulp digestion

2.13.2 Chemical industry

2.13.2.1 Enzymatic production of rhamnose

2.13.2.2 Naringin extraction from kinnow peel waste

2.13.3 Pharmaceutical industry

2.13.3.1 Deglycosylation of flavonoids

2.13.3.2 Preparation of prunin

2.13.3.3 Prodrug therapy

2.1 Yeast

Fungi (Kingdom)
Ascomycota (Phylum)
Archiascomycetes (Class)
Protomycetales (Order)
Protomycetaceae (Family)
Mitosporic Protomycetales (Family)
Saitoella
Schizosaccharomycetales (Order)
Schizosaccharomycetaceae (Family)
Schizosaccharomyces
Euascomycetes (Class)
Oosporidium
Hemiascomycetes (Class)
Saccharomycetales (Order)

Candidaceae	**Dipodascaceae**	**Eremotheciaceae**
Aciculoconidium	*Dipodascus*	*Eremothecium*
Arxula	*Endomyces*	
Botryozyma	*Galactomyces*	
Brettanomyces	*Sporopachydermia*	
Candida	*Stephanoascus*	
Geotrichum	*Yarrowia*	
Kloeckera	*Zygoascus*	
Myxozyma		
Schizoblastosporion		
Sympodiomyces		
Trigonopsis		
Lipomycetaceae	**Metschnikowiaceae**	**Phaffomycetaceae**
Babjevia	*Clavispora*	*Phaffomyces*
Kawasakia	*Metschnikowia*	*Starmera*
Lipomyces		
Smithiozyma		
Zygozyma		
Saccharomycetaceae	**Saccharomycodaceae**	**Saccharomycopsidaceae**
Arxiozyma	*Hanseniaspora*	*Ambrosiozyma*
Citeromyces	*Nadsonia*	*Saccharomycopsis*
Debaryomyces	*Saccharomycodes*	
Dekkera	*Wickerhamia*	
Hansenula		
Issatchenkia		
Kazachstania		
Kluyveromyces		
Kodamaea		
Lodderomyces		
Pichia		
Saccharomyces		
Saturnispora		
Torulaspora		
Williopsis		
Zygosaccharomyces		

Fig. 2.1 Classification of ascomycetous yeast (Barnett *et al* 2000)

Yeasts are groups of unicellular fungi that belong to the phylum Dikaryomycota. Two major groups of yeasts classified are Ascomycetes or Basidiomycetes (Barnett *et al* 2000). The yeasts are characterized by single cells that reproduce by budding from a narrow or broad base (e.g. *Saccharomyces*) or fission from a broad base (e.g. *Schizosaccharomyces*). In addition, pseudohyphae or true hyphae or both may be present (Kurtzman and Fell 1998). Furthermore, during adverse conditions, ascomycetous yeasts are capable of undergoing sexual reproduction that leads to the formation of haploid ascospores of different shapes and nano-scale surface ornamentations. These ascospores, also known as meiospores, are all enclosed within asci. Furthermore, yeasts do not form their sexual states such as asci within or upon fruiting bodies such as apothecia, cleistothecia, etc. (Kurtzman and Fell 1998). Moreover, some ascomycetous yeasts are characterized by the absence of sexual states and these are referred to as anamorphs (Van der Walt and Von Arx 1985).

Ascospores are regarded as haploid cells, which are produced by reduction or meiotic division within an ascus (Yarrow 1998). Ascomycetous yeasts are known to produce ascospores of different shapes (e.g. round, elongate, kidney, needle, hat, saturnoid, walnut, spindle-shaped with a whip like appendage, etc.) and nano-scale surface ornamentations (e.g. smooth, rough, hairy, warty, etc.) all carried within asci (Yarrow 1998). These hyaline spores are produced through the process of amphimictic and automictic sexual reproductive cycles (Van der Walt 1999) that take place within asci. Furthermore, the ascospore number within an ascus can vary significantly, i.e. from one to 150 ascospores and even more. The latter is produced through post meiotic mitosis. In addition, ascospores may be pigmented, sometimes exhibiting yellow, amber, brown or even reddish brown colours (Yarrow 1998). Ascospore morphology, including shape and nano-scale surface ornamentations is an important character in ascomycetous yeast taxonomy. This phenotypic character, known to be conserved especially at genus level, is currently used in the classification of more than 450 ascomycetous yeasts **(Fig. 2.1)** (Yarrow 1998; Barnett *et al* 2000).

The industrial uses of yeasts are not only limited to the traditional processes of making bread, wine and beer **(Table 2.1)** but yeasts are also a rich source of a range of industrially important enzymes such as naringinase, amylase, protease, invertase etc. Another enzyme converts fatty acids to lactones for use in flavourings for margarines and in fruit flavours. Yeast extract has important uses as a source of B and D vitamins, in flavor enhancement and is a common ingredient of routine microbiological culture media.

Table 2.1: Industrial applications of yeast

Yeast cells	Products from yeast cells	Alcohol for drinking	Alcohols from industry
Baker's yeast	Yeast extract	Beer	Industrial alcohol
Dried food yeast	Vitamins B, D	Wine	Gasohol (motor fuel)
Single-cell protein	Enzymes (eg. naringinase, amylase, protease, invertase etc.)	Spirits	Glycerol

2.2 α-L-rhamnosidase

α-L-Rhamnosidase (E. C. 3.2.1.40) cleaves terminal α-L-rhamnose specifically from a large number of natural products which include naringin, rutin, quercitrin, hesperidin, diosgene, ter penyl glycosides and many other natural glycosides containing terminal α-L-rhamnose. The enzyme has wide occurrence in nature and has been reported from animal tissues, plants, yeasts, fungi and bacteria (Yadav *et al* 2010).

2.2.1 Sources of α-L-rhamnosidases

2.2.1.1 Plant α-L-rhamnosidase

Isolation of naringinase has been reported from celery seeds (Hall 1938), grape fruit leaves (Hall 1938; Thomas *et al* 1958; Ting 1958) The enzyme with the name rhamnodiastase, a mixture of α-L-rhamnosidase and β-D-glucosidase, has been reported from *Rhamnus dahurica* (Suzuki 1962). α-L-Rhamnosidase has been studied from the seeds of *Fagopyrum esculentum* (Bourbouze *et al* 1975).

2.2.1.2 Animal α-L-rhamnosidase

The α-L-rhamnosidases from only two animal sources, viz. *Turbo cornutus* liver and pig liver have been reported (Kurosawa *et al* 1973; Qian *et al* 2005).

2.2.1.3 Microbial α-L-rhamnosidase

2.2.1.3.1 Yeast

Some yeasts like *Saccharomyces cerevisiae*, *Hanshula anomala, Debaryomyces ploymorphus* and *Pichia angusta* X349 (Yanai and Sato 2000) show low level of α-L-rhamnosidase activities (McMahon *et al* 1999).

2.2.1.3.2 Bacteia

Bacterial strains producing rhamnosidases are human intestine Bacteroid JY-6 and Fusabacterium K-60 (Jang and Kim 1996; Park *et al* 2005). Thermophilic anaerobic bacterium *Clostridium stercorarium* (Zverlov *et al* 2000), two new thermostable α-L-rhamnosidases from the thermophilic bacterium PRI-1686 (Birgisson 2004), *Lactobacillus* species (Beekwilder *et al* 2009; Avila e*t al* 2009), some *Pseudoalteromonas species* and *Ralstonia pickettii* were obtained from the sea water of sub Antarctic environment also show the α-L-rhamnosidase activities in the low temperature range of -1 to 8 °C (Orrillo *et al*

2007). *Sphingomonas paucimobilis* and *Bacillus* sp GL1 show substantial α-L-rhamnosidase activities in a medium containing gellan as a carbon source (Hashimoto and Murata 1998; Hashimoto *et al* 1999). *Corticium rolfsii* produces α-L-rhamnosidase which is active at low pH (Kaji and Ichimi 1973).

2.2.1.3.3 Fungi

Though some fungal sources are patented and some are kept secrets by the industries, even then α-L-rhamnosidases reported from fungal sources are abundant in literature (Chandler and Nicol 1975; Romero *et al* 1985). Only two commercial preparations of α-L-rhamnosidases, naringinase and hesperidinase are available and both are from fungal sources. Hesperidinase is from *Aspergillus niger* and *Penicillium* species (Monti *et al* 2004) and naringinase is from *Penicillium decumbens* (Romero *et al* 1985). Monti *et al* 2004 have shown the induction of α-L-rhamnosidase production in the fungal strains *Acremonium persicinum* CCF 1850, *Aspergillus aculeatus* CCF 108, *A. aculeatus* CCF 3134, *A. aculeatus* CCF 3138, *A. niger* CCIM K2, *Aspergillus terreus* CCF 3059, *Circinella muscae* CCF 2417, *Emericella nidulans* CCF 2912, *Eurotium amstelodami* CCF 2723, *Fusarium oxysporum* CCF 906, *Mortierella alpina* CCF 2514, *Mucor circinelloides* griseo-cyanus CCIM, *Penicillium oxalicum* CCF 2430, *Rhizopus arrhizus* CCF 100, *Talaromyces flavus* CCF 2686 and *Trichoderma harzianum* CCF 2687 using L-rhamnose, naringin, rutin, hesperidin as inducers. Shanmugam and Yadav 1995 have reported extracellular production of α-L-rhamnosidase by *Rhizopus nigricans*. A number of *Aspergillus* species have been reported for the production of α-L-rhamnosidases (Orejas *et al* 1999; Spagma 2000; Manzanares *et al* 2001; Yadav and Yadav 2001; Yadav and Yadav 2004; Puri *et al* 2005; Koseki *et al* 2008). Feng *et al* 2007 have reported saponin rhamnosidase from *Curvularia lunata*. Scaroni *et al* 2002 have reported some mesophilic fungal strains (viz. *Aspergillus flavus, Mucor racemosus, Fusarium sambucinum, Aspergillus kawachii, Penicillium aureatiogriseum, Trichoderma longibrachiatum, Fusarium solani*) for the production of α-L-rhamnosidases. Hughes *et al* 2004 have characterized an α-L-rhamnosidase from the fungal pathogen of oat leaf, *Stagonospora avenae* which specifically hydrolyses a saponin avenacoside.

2.3 Kinnow production in India

Kinnow is a variety of citrus fruit cultivated extensively in Punjab. It is a hybrid of two citrus cultivars -"King" (*Citrus nobilis*) × "Willow Leaf " (*Citrus deliciosa*), first developed by H. B. Frost at the Citrus Centre of the University of California, Riverside, USA. Kinnow mandarin is quite important as it has a great variety of beverage, industrial and medicinal uses due to its attractive colour, distinctive flavour and being rich source of vitamin 'C', vitamin 'B', β-carotene, calcium and phosphorous (Sogi and Singh 2001). The post-harvest shelf life of kinnow fruit at room temperature is one week and shelf life can be extended to a maximum period of up to 45 days under refrigerated storage conditions.

India stands second in the production of fruits and which accounts for 8% of the world's total fruit production. India holds third rank in respect of production of citrus fruits in the world. The total area under fruit cultivation in Punjab is 0.66 lakh hectares with the production of 11.55 lakh MT. Kinnow, a prevalent citrus fruit in Punjab covering an area of 31,800 hectares with the production of 6.00 lakh tones NHB (2010).

India is the world's second largest producer of fruits (57.73 million tonnes) with its projected value touching 98 Mt by the year 2020-2021 (Banerjee 2009). The citrus occupies an area of about 0.81 Mha with production of 7.50 Mt and yield of 9.26 t/ha and ranks fifth in its production in the world (Anonymous 2010).

2.4 Physicochemical characteristics of kinnow juice

2.4.1 Total sugars

In the citrus fruits, total sugars consist of mainly glucose, laevulose and sucrose. The amount of reducing sugars has been found around 3.95% and that of non reducing sugar is 3.65% (Veldihus 1971). The total sugars content in the juice increased apparently during storage, which might be due to hydrolysis of polysaccharides in to monosaccharide and oligosaccharides.

2.4.2 Total soluble solids

In the citrus fruits, total soluble solids consist of sugar and acids; where in the sugars contribute 75-85% (Jawanda and Singh 1973). Kinnow mandarin has the TSS ranging from 8-15.75% as affected by different storage conditions (Kamaljeet 2002). Total soluble solids content with the increase in storage period was observed in juice of mandarin, sweet orange and lemon by Mehta and Bajaj (1983).

2.4.3 Acidity

Citric acid is the major organic acid in citrus fruits, accumulates in young fruits to 2.5% (w/v) and in the juice before decreasing to about 1% at commercial maturity (Veldihus 1971). In case of kinnow juice, the acidity has been found to range from 0.28%-0.51% (Kamaljeet 2002). There was a significant decrease in acidity content during storage. This might be due to conversion of acids into salts and sugars by enzymes particularly invertase (Kumar *et al* 1992).

2.4.4 Ascorbic Acid

The principle vitamin in the citrus fruits is vitamin C, the amount of which varies with variety, maturity and other factors. Ascorbic acid is relatively stable in citrus products during processing and storage (Vedhius 1971). The ascorbic acid content in kinnow was reported to be in the range of 13.3 to 46.9 mg/100 mL (Pruthi *et al* 1983; Singh *et al* 1978). The ascorbic acid (vitamin 'C') content of the juice decreased during storage with the advancement of storage period, which was probably due to the fact that ascorbic acid being sensitive to oxygen, light and heat was easily oxidized in presence of oxygen by both

enzymatic and non-enzymatic catalyst (Mapson 1970). Similar results were also observed by Ranote and Bains (1982) in kinnow juice. Comparatively lower losses of ascorbic acid was observed in juice samples preserved with higher concentration (750 ppm) of potassium meta-bi-sulphite because higher concentration of potassium meta-bi-sulphite reduced oxidation of ascorbic acid during storage for longer time.

2.5 Bitterness of citrus products

Kinnow juice processing and commercial utilization has faced a great hindrance due to the development of bitterness. The intense bitterness development within few hours of juice extraction renders the juice unpalatable and thus processing of juice in beverages, juice concentrate or juice powder is highly limited (Puri *et al* 1996).

2.5.1 Flavonoids bitterness

Citrus fruits and its tissues have many flavonoids like hesperidin, limonin, naringin. These flavonoids is present in oranges, grapefruit, lemon, these flavonoids may cause interference during the citrus fruit juice processing and cause for the bitter taste (Konno *et al* 1982). Naringin is the major component in grapefruit with very bitter taste and a threshold of 20 mg Kg^{-1} in water and detectable limit less than 1.5 mg Kg^{-1} (Chandler and Nicol 1975).
Bitterness is a common flavor characteristic in the fruit of some species of the citrus genus and is determined by the concentration and composition of branched-chain flavanone glycosides, the prevailing flavonoids in citrus (Harborne 1967; Berhow *et al* 1998; Gattuso *et al* 2007). The bitter flavanone 7-*O*-neohesperi-dosides (e.g. neohesperidin and naringin) are the dominant and in some cases the only flavanone glycosides in bitter citrus species (i.e. pummelo, grapefruit and bitter orange), and comprise the branched-chain disaccharide neohesperidose (rhamnose-2-*O*-glucose) *O*-linked to position 7 of the flavanone (Horowitz and Gentili 1961; Jourdan *et al* 1985; Peterson *et al* 2006a). The tasteless 7-*O*-rutinosides (e.g. hesperidin and narirutin) are the only flavanone glycosides in non-bitter citrus species (i.e. sweet oranges, mandarins, clementine, citron and lemon), and comprise the branched-chain disaccharide rutinose (rhamnose-6-*O*-glucose) *O*-linked to position 7 of the flavanone (Hall 1925; Rousseff *et al* 1987; Peterson *et al* 2006b). Beyond the effect on fruit flavor, it is assumed that flavanone glycosides have a role in protecting young citrus tissue against disease (Del Rio *et al* 2004), because they accumulate to very high concentrations in young tissue (mainly leaves and fruit) and are gradually diluted during continued development (Jourdan *et al* 1985; Castillo *et al* 1992; Bar-Peled *et al* 1993; Ortuno *et al* 1995).

2.5.2 Limonoid bitterness

Limonoids, the bitter constituents of orange juice, are a group of highly oxygenated triterpenoids observed in the Rutaceae, Meliaceae and related plant families. Limonin is a major component of limonoids in citrus and lowers the market value of fruit juice. This bitterness has been referred to as ‘delayed bitterness’ (Mayer and Beverly 1968). The

mechanism for generating limonin is ascribed to the conversion of a nonbitter precursor, limonoate A-ring lactone (LARL), to limonin under the acidic condition in the juice. This reaction is accelerated by the activation of limonoid D-ring lactone hydrolase (Mayer *et al* 1969). Furthermore, if orange fruits are frozen or mechanically damaged, the conversion from LARL to limonin will occur in the citrus fruit itself. Contrary to navel orange, Satsuma mandarin (*C. unshiu* Marc.) fruits, the most popular citrus fruit in East Asia, are less bitter, even from the early to mid developmental stages (Hashinaga *et al* 1977), mainly because of the conversion of LARL to the tasteless limonin glucoside (Ozaki *et al* 1995). Although the molecular regulation for this conversion is not yet fully understood, the gene encoding UDP-D-glucose:limonoid glucosyltransferase which catalyzes the conversion of LARL to limonin glucoside was recently isolated from Satsuma mandarin (Kita *et al* 2000) based on information of the purified enzyme protein (Hasegawa *et al* 1997).

2.6 Debittering of citrus fruit juices

2.6.1 Physicochemical approaches

2.6.1.1 Adsorptive debittering

The use of polyamides to selectively adsorb significant quantities of limonin from Washington navel orange juice has been explored successfully (Chandler and Kefford 1968; Griffith 1969). Similarly, the uses of a variety of adsorbents such as cellulose acetate, nylon-based matrices, porous polymers and ion exchangers have been explored to reduce bitterness and acidity in grapefruit juice (Johnson and Chandler 1988). Various degrees of debittering have been achieved with individual matrices and their combinations and cellulose acetate and two porous polymers proved to exhibit intense affinity for limonin.

2.6.1.2 Chemical methods

Treatments with ethylene (20 pg mL^{-1}) for 3 h to accelerate ripening (limonin catabolism) in navel oranges, lemons and grapefruits with a concomitant reduction in bitterness have been reported (Maier *et al* 1973). Using carbon dioxide at pressures of 21 to 41 MPA at 30 to 60 °C for 1 h resulted in an average removal of 25% of the limonin from navel orange juice. By extending the treatment to 4 h, 60% of the limonin was removed (Kimball and Seth 1987).

2.6.1.3 Debittering by passage through polystyrene- DVB resins

In batch operations, cross-linked divinyl benzene-styrene resin reduced naringin and limonin content in grapefruit juice by 80 and 90%, respectively (Puri 1984); the resultant juice was less bitter. Laboratory studies on the effect of mean pore diameter, percent cross-linkage and specific area of polystyrene DVB resins for the adsorption of limonin and naringin from grapefruit juice have been reported (Manlan *et al* 1990). Porcessing navel orange juice with these resins had no effect on the minerals, acid, and amino acids content of the juice (Kimball and Norman 1990).

2.6.1.4 Debittering by β- cyclodextrin

Soluble 0.5% β-cyclodextrin has been used to reduce 58% of the initial bitter taste of juice from grapefruit, Iyo orange and C. *natsudaidai* (Konno *et al* 1982). The authors concluded that reduced bitterness was due to formation of an inclusion complex between k-cyclodextrin and naringin or limonin. This information led to utilization of a β-cyclodextrin polymer in a batch/continuous column mode to remove limonin and naringin from their aqueous solutions, orange and grapefruit juices (Shaw and Wilson 1983).

Limitations

The methods altered the chemical composition of the juice either through chemical reactions or removal of nutrients, flavor and color etc. (Puri *et al* 1996).

2.6.2 Biotechnological approaches

The naringin content of juices can be reduced by several chemical technologies, but these methods may alter the composition of juice, may affect organoleptic properties and the quality of the juice, for the removal of nutrients, flavour and colour components. Therefore, acid hydrolysis is not suitable to commercial processes (Abbatea *et al* 2012).

The enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency, and a convenient operation for removing the bitterness in large-scale commercial production (Yadav *et al* 2010). Naringin can be hydrolyzed by the α-L-rhamnosidase activity into rhamnose and prunin (4,5,7-trihydroxyflavonone-7-glucopyranoside), in which prunin can be further hydrolyzed by the β-D-glucosidase activity into glucose and naringenin (4,5,7-trihydroxyflavonone) (Chien *et al* 2001). Due to the lesser bitterness of prunin than that of naringin about one third, only activity of the α-L-rhamnosidase is essential. Hence, it is of commercial interest that the flavors should be more acceptable by the consumer (Norouzian *et al* 1999).

2.7 Beverages

2.7.1 Ready to serve beverages

Citrus fruits are considered to be the rich source of ascorbic acid, pectin, carotenes, citric acid, and minerals like calcium and phosphorous. Consumption of high sugar drinks lead to various diseases such as diabetes, obesity and dental caries. Ahmed *et al* 2008 studied the formulation of diet beverage with various combinations of intense sweeteners and compared with control beverage containing sucrose. Physiochemical changes in beverage were also investigated at various storage intervals. A gradual increase in reducing sugar level was observed in all treated samples with the passage of time, while non-reducing sugars decreased gradually during storage studies. The declining trend in ascorbic acid contents of mandarin drink was increased as a function of storage. Sensory results showed that there was declining trend in the scores obtained for color. The overall results showed that combination of different sweeteners gave best results for taste than without combinations.

2.7.2 Blended beverages

Different fruit juice blends were prepared as (kinnow juice: aonla juice: ginger juice in 100: 0: 0, 95: 5: 0, 92: 5: 3 ratio and kinnow juice: pomegranate juice: ginger juice in 90: 10: 0, 87: 10: 3 ratio) for improving flavour, palatability, nutritive and medicinal value. Addition of ginger juice in blends improves the quality and reduces microbial growth. The juice was acceptable after six months of storage at room temperature (Bhardwaj and Mukherjee 2011).

Different fruit juice blends were prepared as (kinnow juice: aonla juice: ginger juice pomegranate juice for improving flavor and nutritive value. The juice blends were preserved by pasteurization (75 °C or 85 °C for 15 min.) and by addition of potassium meta-bi-sulphite (500 or 750 ppm). These blends were stored in 200 mL colourless glass bottles at (4±1°C) and tested at three months interval upto six month. Blends were examined for physico-chemical, sensory evalution and microbial population. The individual effect of juice blending ratio, processing temperature and potassium meta-bi-sulphite treatment was found to be significant in prolonging storage life and maintaining the acceptable quality. The juice blends processed at 75 °C for 15 min with 750 ppm potassium metabi- sulphite was the most effective treatment. However, a minimum microbial population was recorded with juice processed at 85 °C temperature (Bhardwaj 2013).

2.7.3 Alcoholic beverages

Two different strains of *Saccharomyces cerevisiae* NCIM 3095 and NCIM 3287 were evaluated in the production of guava fruit wine. Guava must concentrations were adjusted to 22 °Brix with sucrose solution and batch fermentations were performed. For optimization of guava wine fermentation various parameters, such as the osmotolerance, alcohol tolerance, inoculum size, Initial pH of the medium, amount of SO_2, amount of diammonium phosphate and Incubation temperature were studied for both the strains. For guava wine production *Saccharomyces cerevisiae* NCIM 3095 gave much better results as compare to *Saccharomyces cerevisiae* NCIM 3287 (Sevda and Rodrigues 2011).

2.7.4 Carbonated beverages

A reliable, controllable, simple, and reproducible technology from astringent fruits has been developed for the production of low- alcoholic naturally carbonated beverage with improved tangy taste, appearance, aroma, extended shelf life, and retention of all the nutrients. The yeast on inoculation @ 0.5% in astringent in amla juice (13%), TSS adjusted to 16.0 °B, and fermentetion at 20 ± 5 °C for 36 h produces a new low- alcoholic naturally carbonated beverage. The physicochemical parameters of freshly prepared beverage juice 13%, pH 3.0, TSS 16.0 °B, acidity 0.38%, Brix acid ratio 42.10, ascorbic acid 120.0 mg/100 mL. The physicochemical parameters did not change significantly during storage. The volatile components like propanol, butanol, acetaldehyde, methanol, ethyl acetate, and isopropanol

were found to be absent while the percentage of ethanol was 1.16% after three months of storage. Shelf life of the beverage is three months under refrigerated conditions (4 °C) (Jairath *et al* 2012).

A technology to produce low alcoholic self carbonated beverage from carrot-lemon juice was developed. It is a reliable, controllable, reproducible technology, and especially safeguards the interest of horticulturists during seasonal glut of the fruits. The physicochemical characteristics of freshly prepared fermented carrot-lemon beverage (3:1) TSS 16 ºB, pH 3.7, acidity 0.36%, brix acid ratio 44.44, ethanol 0.4% (w/v), CO_2 0.9 bar and viable cell count was $1x10^7$ cfu mL^{-1}. The physico-chemical changes recorded after storage for three months at refrigerated temperature showed TSS 13 °B, pH 3.3, Brix acid ratio 26.53, acidity 0.49%, ethanol 0.8% (w/v), CO_2 1.5 (bar) and viable cell count (cfu mL^{-1}) was $9.5x10^8$ cfu mL^{-1}. Naturally produced CO_2 during fermentation adds effervescence, sparkle, tangy taste to the beverage. CO_2 has antimicrobial properties and shelf life of beverage is three months (Sahota *et al* 2010a).

A pure yeast isolate from whey beverage, phenotypically and molecularly characterized, was used for the preparation of low alcoholic naturally carbonated beverage from guava var. *Allahabad Safeda, Lucknow-49, Punjab Pink* and its blends with lemon var. *Baramasi* under optimized fermentation conditions. The specific growth rate (h^{-1}) and generation time (h) of yeast in guava: lemon (1:1) beverage was 0.35 and 1.93 respectively (Sahota *et al* 2010b).

A process for preparation of carbonated acidic whey beverage by blending with different types of fruit juices at various levels of concentration has been standardized. The acidic whey (shrikhand whey) beverage base was blended with three different types of fruit juices i.e. mango, orange and pineapple at different levels of concentrations i.e. 18.0, 20.0, 22.0 and 24.0%. The unclarified and prefiltered acidic whey (shrikhand whey) beverage base with 22% unclarified mango juice, orange juice and pineapple juice concentration scored higher overall acceptability i.e. 8.23, 7.96 and 8.26 respectively. The ultrafiltered acidic whey (shrikhand whey) beverage base with 22% pineapple juice was found to be the best among all prepared beverage. The selected beverages were carbonated at three different levels of carbonation i.e. 25, 30, 35 psi at 4±1°C temperature and were subjected to organoleptic evaluation. The unclarified mango beverage scored higher i.e. 8.33 at 25 psi. The prefiltered orange flavoured beverage scored higher i.e. 8.10 at 35 psi. The ultrafiltered pineapple flavoured beverage scored highest i.e. 8.53 at 30 psi, for overall acceptability than that of unclarified mango and prefiltered orange flavoured acidic whey (shrikhand whey) beverages (Dilipkumar and Yashi 2014).

2.8 Microbial production of α-L-rhamnosidase enzyme

Microbial rhamnosidase mainly produced by submerged fermentation process. The highest enzyme titer of 9.68 U of enzyme per milliliter was achieved using a step-wise addition of small amounts of naringin up to 7 days of fermentation in a complex medium containing molasses, peptone, and salts (Kumar 2010). Reported inducers for naringinase production are rhamnose (Thammawat *et al* 2008), hesperidin (Fukumoto and Okada 1973), naringin (Bram and Solomons 1965; Puri *et al* 2008) and citrus peel powder (Puri *et al* 2011). Rajal *et al* 2009 reported that the production of biomass of *Penicillium ulaiense* using rhamnose as both substrate and an inductor for enzyme production was rapid, with no lag phase, indicating that the inoculum was in good condition. The maximum concentration of biomass, 3.2 g L^{-1}, was reached on the third day of culture. The stationary phase for biomass production began when the rhamnose in the medium was exhausted. After the fifth day of culture, changes were observed macroscopically as a progressive darkening in the medium and microscopically by the increase of sporulation. The production of enzymes was negligible for the first 2 days. Then, the production rate increased rapidly, especially for β-glucosidase, and the activity remained almost constant up to around 8-9 days of culture. The maximum α-L-rhamnosidase activity obtained in the batch reactor was the same as for the exponential phase, and YR/S = 17.8 IU g^{-1} and YG/S = 197 IU g^{-1} for enzymatic production. The maintenance coefficient for the stationary phase, determined from the mass balance for substrate, was mS = -0.0027 g g^{-1} d^{-1} (Rajal *et al* 2009).

Elinbaum *et al* (2002) mentioned that naringin is a better inducer than rhamnose in the production of naringinase using *A. terreus* in solid fermentation. Furthermore, they suggest that solid fermentation gives better results in the production of naringinase than its liquid counterpart as reported too by Viniegra *et al* (2003) where shown effective growth of fungi and production of enzymes in solid-state fermentation (SSF) in comparison to liquid cultivation.

2.8.1 Effect of carbon sources on rhamnosidase production

Koseki *et al* 2008 suggested that the carbon source clearly influences the inducible production of extracellular α-L-rhamnosidase by *A. kawachii*. The α-L-rhamnosidase activity was the highest with 0.5% L-rhamnose. However, on day 5, the α-L-rhamnosidase activity was practically the same with 0.5% L-rhamnose and 0.5% L-rhamnose supplemented with 1% D-glucose. On the other hand, the α-L-rhamnosidase activity did not occur with 0.5% D-glucose and 0.5% wheat bran. The production of α-L-rhamnosidase by *A. nidulans* is mediated by carbon catabolite repression, which appears to be CreA-independent (Orejas *et al* 1999). When *A. kawachii* was grown on 0.5% L-rhamnose, α-L-rhamnosidase production was significantly induced. On the other hand, the production was repressed when *A. kawachii* was grown on 0.5% L-rhamnose supplemented with 1% glucose. The enzyme was not produced

when *A. kawachii* was grown on 0.5% glucose as the sole carbon source.

Chen *et al* 2010 reported that after 5 days of fermentation, naringin exhibited the highest naringinase activity compared to all other carbon sources ($P < 0.05$). Pomelo pericarp powder produced comparable naringinase activity and was not significantly different from naringin. Relative activities of other carbon sources to naringin were: pomelo pericarp powder, 99.71%; maltose, 86.49%; rhamnose, 84.93%; pomelo juice, 66.57%; lactose, 54.78%; sucrose, 39.86%; and glucose, 28.74%. Since pomelo pericarp powder is a cheap resource and has additional environmental benefits (pomelo pericarp powder is a waste of pomelo juice production), it was selected for medium formulation in subsequent experiments. Subsequently, a trial was conducted to determine optimum pomelo pericarp powder concentration. Naringinase activity was increased with increased pomelo pericarp powder from 5 to 15 g L^{-1} and then decreased sharply from 15 to 35 g L^{-1} with the highest activity at 15 g L^{-1}.

α-L-Rhamnosidase production from *Pseudoalteromonas* sp. 005NJ, several monosaccharides as carbon sources were assayed, individually or combined, and evaluated after 6 and 24 h of cultivation. D-fructose enhanced enzyme activity threefold, reaching a plateau of maximum specific activity between 0.2-0.4 M of this monosaccharide (Mazzaferro *et al* 2008).

2.8.2 Effect of nitrogen sources on rhamnosidase production

In general, in comparison with organic nitrogen sources, inorganic nitrogen sources usually yielded relatively low naringinase production in shaking-flask cultures. These results are in agreement with those of submerged cultures of fungi reported by Norouzian *et al* (2000). It has been suggested that inorganic nitrogen sources could only marginally synthesize certain essential amino acids in fermentation by fungi and organic nitrogen sources were favorable for metabolite production (Hwang *et al* 2003; Kim *et al* 2003).

Different nitrogen sources were added at 10 g L^{-1} to replace potassium nitrate and ammonium sulphate in the base medium in a shaking flask culture of *A. oryzae* JMU316. Among all nitrogen sources, peptone was the most effective and was significantly better than the control ($P < 0.05$). In general, organic nitrogen sources yielded higher naringinase activity than inorganic nitrogen sources in liquid culture. Compared to the control, relative activities were: peptone, 106.40%; soybean meal, 96.19%; beef extract, 93.31%; NH_4Cl, 74.86%; $NH_4H_2PO_4$, 61.60%; casein, 48.90%; and NH_4NO_3, 31.76% (Chen *et al* 2010).

2.9 Optimization of α-L-rhamnosidase production using response surface methodology

Response surface methodology was used to optimize the fermentation medium for enhancing naringinase production by *Staphylococcus xylosus*. The first step of this process involved the individual adjustment and optimization of various medium components at shake flask level. Sources of carbon (sucrose) and nitrogen (sodium nitrate), as well as an inducer

(naringin) and pH levels were all found to be the important factors significantly affecting naringinase production. In the second step, a 22 full factorial central composite design was applied to determine the optimal levels of each of the significant variables. A second-order polynomial was derived by multiple regression analysis on the experimental data. Using this methodology, 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), respectively. Under optimal conditions, the experimental naringinase production was 8.45 U mL^{-1}. The determination coefficients (R_2) were 0.9908 and 0.9950 for naringinase activity and biomass production, respectively, indicating an adequate degree of reliability in the model (Puri *et al* 2010a).

Mukund *et al* 2013 applied response surface modeling based on central composite design to determine the effects of three independent variables (sucrose, yeast extract and naringin) and their mutual interactions. In total, 20 experiments were conducted and a statistical model was developed, which predicted naringinase production of 10.61U L^{-1} from *Bacillus methylotrophicus*. Subsequently, verification experiments were conducted and validity of the model was verified. Bioreactor studies conducted with the optimized medium showed an enzyme production of 12.05U L^{-1} from *Bacillus methylotrophicus* within 34 h of fermentation.

2.10 Purification of α-L-rhamnosidase enzyme

The α-L-rhamnosidase from the liver of *T. cornutus*, a marine gastropod, has been purified to homogeneity using column chromatography with CM cellulose and Sephadex G-150, heat treatment, freezing and thawing in acidic pH (Kurosawa *et al* 1973). The purification of α-L-rhamnosidase from pig liver involved extraction of the enzyme by homogenizing pig liver with buffer, fractional precipitation with ammonium sulphate, dialysis and ion exchange chromatography on DEAE-cellulose (Qian *et al* 2005). The purification of α-L-rhamnosidase from the seeds of *F. esculentum* involved extraction of the crude enzyme, fractionation by ammonium sulphate precipitation and chromatography on columns of Sephadex G-75, DEAE-Sephadex and Ultrogel AcA-44 and has been found to be pure according to the criteria of discgel electrophoresis (Bourbouze *et al* 1976).

The α-L- rhamnosidase from the human intestinal bacterium *Bacteroides* JY-6 has been purified by disrupting the bacterial cells suspended in 20mM phosphate buffer of pH 7 by ultrasonicator, fractionating the resultant extract by ammonium sulphate precipitation, column chromatography on DEAE-cellulose, Silica- PAE, Sephacryl S-300 and hydroxyapatite, respectively (Jang and Kim 1996). The α-L-rhamnosidase from another human intestinal bacterium *Fusobacterium* K-60 has been purified using a method which involved disruption of bacteria by ultrasonicator, fractionation of the protein by ammonium sulphate precipitation and column chromatography on Butyl-Toyopearl, hydroxyapatite,

Sephacryl S- 300 and Q-Sepharose (Park *et al* 2005).

Hashimoto *et al* (1999) have purified α-L-rhamnosidase of *Bacillus* sp. GL1 by extracting the enzyme after disrupting the cells by ultrasonication, ammonium sulphate precipitation and column chromatography on DEAE-Sepharose CL- 6B, Butyl-Toyopearl 650M, Sephacryl S-200HR and QAE-Sephadex A-25. Miake *et al* (2000) have purified and characterize an intracellular α-L-rhamnosidase from *Pseudomonas paucimobilis* FP2001. The thermo stable α-L-rhamnosidase Ram A of *C. stercorarium* has been purified and characterized by Zverlov *et al* (2000). Beekwilder *et al* (2009) have purified α-L-rhamnosidase from *Lactobacillus plantarum* and *Lactobacillus acidophilus*. Avila *et al* (2009) have over expressed two α-L-rhamnosidase genes from *L. plantarum* NCC 245 in *Escherichia coli* and have purified it.

Roitner *et al* (1984a) have tried to characterize α-L-rhamnosidase and β-D-glucosidase activities from the commercial preparation of naringinase of *A. niger* origin. Two α-L-rhamnosidase with different substrate specificities have been isolated from a commercial preparation produced by *A. aculeatus* by Mutter *et al* (1994). The first was active towards p-nitrophenyl α-L-rhamnopyranoside, naringin and hesperidin. The second α-L-rhamnosidase was active towards rhamnogalacturonan (RG fragments) releasing rhamnose. Soria *et al* (1999) have purified α-L-rhamnosidase from the culture filtrate of *A. terreus* CECT-2663 grown on medium containing either rhamnose or naringin as a carbon source. They were successful in separating β-D-glucosidase activity from α-L-rhamnosidase activity when rhamnose was inducer. α-L-rhamnosidase from *Aspergillus nidulans* CECT 2544 has been purified from the culture filtrate of the fungal strain grown on L-rhamnose as the sole carbon source by the combination of batch adsorption on DEAE A-50, two steps of Hi Load 16/10 Q Sepharose FF column, Hi Load 26/S Sepharose FF column and finally by gel filtration on Superose 12 HR 10/30 column (Manzanares *et al* 2000). Manzanares *et al* (2001) have purified and characterized two different α-L-rhamnosidases RhaA and RhaB from the culture filtrate of *A. aculeatus* grown on hesperidin using cation exchange and gel filtration chromatography. Purification and characterization of ginsenoside α-L-rhamnosidase from fungus *Absidia sp.* (EECDL-39) has been reported by Yu *et al* (2002).

Feng *et al* (2007) have purified and characterized a saponin active rhamnosidase from *C. lunata* using ammonium sulphate precipitation, gel filtration, cation and anion exchange chromatography. Purification and characterization of naringinase from *A. niger* MTCC 1344 has been reported by Puri and Kalra (2005). The purification involved concentration of the culture filtrate by ultrafiltration, precipitation by ammonium sulphate, ion exchange chromatography on Q Sepharose and gel filtration on Sephadex G-200. Koseki *et al* (2008) have purified an α-L-rhamnosidase from the culture filtrate of *A. kawachii* grown on L-rhamnose as a sole carbon source using fractional precipitation by ammonium sulphate,

HPLC on ion exchange and gel filtration columns.

2.11 Characterization of purified α-L-rhamnosidase enzyme

2.11.1 Effect of pH

The stability of an enzyme is affected by many factors, such as temperature, pH, oxidative stress, solvent, binding of metal ions or cofactors and the presence of surfactants (Eijsink *et al* 2005). Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke 1990).

Rojas *et al* 2011 reported that the *A. luteo albus* Rhase exhibits optimal activity at pH 8.0 towards PNP-Rha as substrate, which is quite different from those of *Aspergillus Rhases* (Manzanares *et al* 1997; Mutter *et al* 1994; Roitner *et al* 1984b). Most of the fungal Rhases described in the literature have maximal activity at acid pH values when using PNP-Rha as substrate. At pH 9.5, *A. luteo albus* Rhase retained more than 67% of its maximum activity, and 62% of its maximum activity at pH 11. A broad pH optimum is preferable for applications of Rhase in the food and pharmaceutical industries. The activity of Rhase under alkaline conditions where flavonoids such as naringin and hesperidin are highly soluble is remarkable. Therefore, its potential application for the production of L-rhamnose and the corresponding glycosylated flavanone should be considered.

Rajal *et al* 2009 reported that the optimum pH (5.0) of *P. ulaiense* α-L-rhamnosidase was in the range reported for this enzyme produced by other fungi, between 3.4 and 6.5 (Romero *et al* 1985; Puri *et al* 1996; Soria *et al* 1999; Spagna *et al* 2000; Scaroni *et al* 2002). In that range of pH the activity was more than 60% of its maximum value, showing a great potential for α-L-rhamnosidase applications as an enhancer of wine aroma (Spagna *et al* 2000). Other previously reported properties of the *P. ulaiense* α-L-rhamnosidase such as the absence of mycotoxins in the culture filtrate (Rajal 2002) further encourage future studies on use of this enzyme in food technology. Yanai and Sato (2000) reported that the enzyme from yeast *Pichia angusta* showed optimum activity at pH 6. *P. paucimobilis* FP2001 and *Sphingomonas* sp. R1 (Hashimoto and Murata 1998) Rhase had pH optimums at slightly alkaline pH. Manzanares *et al* 2001 found that the optimal pH of the purified RhaA and RhaB was to be 4.5 to 5 in McIlvaine buffer. RhaA and RhaB showed more than 85% of their maximum activity in the pH ranges of 4 to 5.5 and 3 to 5.5, respectively. Both α-L-rhamnosidase activities were stable in the pH range from 3 to 5. After 20 h of incubation at 30 °C, the enzymes retained 80% (RhaA) and 90% (RhaB) of their initial activities over the same

pH range. The *A. nidulans* α-L-rhamnosidase showed a broad pH optimum in the range 4.5±6, similar to most of the *Aspergillus* α-L-rhamnosidases described (Roitner *et al* 1984a; Mutter *et al* 1994; Manzanares *et al* 2000; Gallego *et al* 2000). At pH 4 and pH 7 it retained more than 95% of its maximum activity, and 55% maximum activity at pH 3.5. The enzyme was stable at pH 4.5 after 24 h of incubation, whereas at pH 4 it retained 40% of its activity and no activity could be detected at pH 3 and 3.5. This may explain the loss of α-L-rhamnosidase activity after the purification on S-Sepharose FF at pH 3.8 (Manzanares *et al* 2000). The enzyme from *Aspergillus kawachii* exhibited optimal activity at pH 4.0 (Koseki *et al* 2008). The pH optima of the purified enzyme were found to be 7.0 using p-nitrophenyl-α-L-rhamnopyranoside as the substrate (Yadav *et al* 2012). Most of the α-L-rhamnosidases reported so far have pH optima either in the acidic (Kurosawa *et al* 1973; Manzanares *et al* 2001) or in the basic (Zverlov *et al* 2000; Hashimoto and Murata 1998; Miake *et al* 2000) pH range. Only α-L-rhamnosidases of *Bacteroides* JY-6 (Jang and Kim 1996) pig liver (Qian *et al* 2005) and *Bacillus* sp. GL1 (Hashimoto *et al* 1999) have pH optima in the neutral pH range. The purified α-L-rhamnosidase is another enzyme having pH optimum in the neutral pH range, which will be more suitable for the enhancement of wine aroma (Yadav *et al* 2012).

2.11.2 Effect of temperature

The optimal temperature for the activity of the α-L-rhamnosidase from various species of *Aspergillus* has been reported to range from 40 °C to 65 °C (Manzanares *et al* 2001; Orejas *et al* 1999; Gallego *et al* 2001). The enzymes isolated from *A. niger* and *A. aculeatus* were found to retain 85% and 87% of their original activity following heat treatment for 4 h at 50 °C and 55 °C, respectively (Manzanares *et al* 2001). On the other hand, the enzyme from *A. nidulans* is extremely unstable at 60 °C and loses 90% of its activity following incubation at this temperature for 1 h (Orejas *et al* 1999). It has been reported that protein glycosylation plays a role in the thermostability of hydrolytic enzymes, including the GHs from the various species of *Aspergillus* (Jafari- Aghdam *et al* 2005; Koseki *et al* 2006). The hyperglycosylation of Ak-RhaA may be involved in them thermostability.

Yadav *et al* 2012 reported that the temperature optimum of the enzyme from *Penicillium citrinum* MTCC-8897 is 60 °C, which is in the range 40-80 °C reported for other α-L-rhamnosidases in the literature (Yanai and Sato 2000; Scaroni *et al* 2002; Miake *et al* 2000). The maximum activity of the α-rhamnosidase from *Penicillium ulaiense* was observed at temperatures between 60 and 60 °C, above that range the activity was highly sensitive to very small increases in temperature, and 90% of the activity at 65 °C was lost at 75 °C (Rajal *et al* 2009).

Rojas *et al* 2011 found that rhamnosidase enzyme *Acrostalagmus luteo albus* was stable up to 40 °C for 4 h, whereas it retained 95% of its original activity after 10 h of

Table 2.2: Sources of α-L-rhamnosidases along with some properties of the enzyme (Yadav *et al* 2010)

Sr. No.	Source	pH optima	Temperature optima (°C)	Molecular weight	pI	Reference
1	*Fagopyrum esculentum*	–	–	70,000[a]	3.7	Bourbouze *et al* 1976
2	*Turbo cornutus*	2.8	–	–	–	Kurosawa *et al* 1973
3	*Pig Liver*	7	42	47,000[b]	–	Qian *et al* 2005
4	*Bacteroid JY-6*	7	–	120,000[b], 240,000[a]	4.2	Jang ang Kim 1996
5	*Fusabacterium K-60*	5.5	–	41,000[b], 170,000[a]	5.2	Park *et al* 2005
6	*Clostridium stercorarium*	7.5	60	–	–	Zverlov *et al* 2000
7	*Pseudoalteromonas species*	6.0	40	–	–	Orrillo *et al* 2007
8	*Ralstonia pickettii*	–	–	–	–	Orrillo *et al* 2007
9	*Bacillus sp GL1*	7	50	100,000[b],c	–	Hashimoto *et al* 1999
10	*PRI-1686 (RhmA & RhmB)*	7.9, 5–6.9	70	104,000[b], 210,000[a] and 107,000[b], 210,000[a]	–	Birgisson *et al* 2004
11	*Lactobacillus plantarum NCC 245 (RhaB1and RhaB2)*	7 and 5	50 and 60	73,000[b], 155,000[c], and 57,000[b], 100,000[c]	–	Avila *et al* 2009
12	*Lactobacillus plantarum*	7	–	–	–	Beekwilder *et al* 2009
13	*Lactobacillus acidophilus*	6	–	–	–	Beekwilder *et al* 2009
14	*Corticium rolfsii*	2	–	–	–	Kaji and Ichimi 1973
15	*Pseudomonas paucimobilis FP2001*	7.8	45	112,000[b],a	7.1	Miake *et al* 2000
16	*Pichia angusta X349*	6	40	88,000[b], 90,000[c]	4.9	Yanai and Sato 2000

17	*Rhizopus nigricans*	6.5	60–80	–	–	Shanmugam and Yadav 1995
18	*Aspergillus aculeatus* (RhaA and RhaB)	4.5–5.0	–	92,000[b] and 85,000[b]	–	Manzanares *et al* 2001
19	*Aspergillus aculeatus* (pnp rhamnohydrolase and RG-rhamnohydrolase)	5.5 and 4	60	87,000[b] and 84,000[b]	–	Mutter *et al* 1994
20	*Aspergillus nidulans*	4.5–6	60	102,000[b]	5	Manzanares *et al* 2000
21	*Aspergillus terreus*	5.5	60	89,000[b], 97,000[a]	–	Sarconi *et al* 2002
22	*Aspergillus flavus*	6.5	50	–	–	Sarconi *et al* 2002
23	*Mucor racemosus*	5.5–6.5	55–60	–	–	Sarconi *et al* 2002
24	*Fusarium sambucinum*	5.5–6.5	55–60	–	–	Sarconi *et al* 2002
25	*Aspergillus kawachii*	4.5	60	–	–	Sarconi *et al* 2002
26	*Penicillium aureatiogriseum*	–	60	–	–	Sarconi *et al* 2002
27	*Trichoderma longibrachiatum*	4.5–5.5	60	–	–	Sarconi *et al* 2002
28	*Fusarium solani*	6.5	–	–	–	Sarconi *et al* 2002
29	*Curvularia lunata*	4	50	66,000[b]	–	Feng *et al* 2007
30	*Absidia sp.*	5	40	53,000[b]	–	Yu *et al* 2002
31	*Aspergillus niger*	4	50	168,000[a]	–	Puri and kalra 2005
32	*Aspergillus kawachii*	4	50	90,000[b]	–	Koseki *et al* 2008
33	*Clavispora lusitaniae*	4	50	85000[b]	-	Present study

a. Molecular weight determined by gel filtration.

b. Molecular weight determined by SDS-PAGE.

c. Molecular weight determined by native PAGE.

incubation, even when no protective agent was added. At its optimum temperature (55 °C), the enzyme retained 81% of its initial activity after 4 h of incubation. Temperatures higher than 55 °C resulted in a rapid loss of enzyme activity **(Table 2.2)**.

2.11.3 Effect of enzyme and substrate concentration

The substrate specificity of α-L-rhamnosidase for various α-L-rhamnose-containing disaccharides was examined by determining their first-order rate constants calculated from the Kézdy-Swinbourne plot (Cornish-Bowden 1976).

The Km values for p-nitrophenyl-α-L-rhamnoside are in the range 0.057-2.8 mM, for naringin 0.021-1.9 mM, for hesperidin 0.02-1.33 mM, for rutin 0.028-1.44 mM, for quercitrin 0.077-0.89 mM and for poncirin 0.02-0.93 mM. Majority of α-L-rhamnosidases are active on α-1, 2 glucosidic linkages whereas the number of α-L-rhamnosidases active on α-1, 6 linkages comes second. There are some α-L-rhamnosidases active on α-1, 4 linkage but α-L-rhamnosidases active on other glycosidic linkages are rare. The glucoamylase from *C. lunata* having steroidal saponin rhamnosidase activity hydrolyses a large number of spirostanoside and furostanoside (Yadav *et al* 2010).

The enzyme naringinase from *Candida tropicalis* hydrolyze both the substrates rutin (0.1%) and naringin (0.1%). The extent of hydrolysis of naringin was more than rutin (Saranya *et al* 2009). The enzyme obtained from the *Aspergillus niger* was more specific towards the naringin than rutin (Puri *et al* 2005).

The hydrolysis rate of 2.56 mM naringin at pH 5.0 and 50 °C, determined using regression analysis, was 1.6 ± 0.2 μmol min^{-1} mL^{-1}. The hydrolysis of p-NPR at the same concentration was 3.4 μmol min^{-1} mL^{-1}, twice the value obtained using naringin, a more complex molecule (Rajal *et al* 2009). Other reported studies performed with the *P. ulaiense* α-rhamnosidase showed that it has a special ability to hydrolyze the insoluble substrate hesperidin, but not naringin (Scaroni *et al* 2002).

The rhamnosidase enzyme from *Acrostalagmus luteo albus* was active towards naringin, in which the L-rhamnose residue is α-1,2 linked to the β-D-glucoside; towards hesperidin which has an α-1,6 linkage to the β-D-glucoside; and also towards quercitrin, where the L-rhamnose residue is linked directly to the aglycon. This enzyme is able to hydrolyze α-1,2 and α-1,6 linkages to β-D-glucosides in addition to direct linkages from the L-rhamnose residue to the aglycon. They also quantified the initial and final amounts of substrates and reaction products by HPLC, and hydrolysis percentages were calculated as follows: hesperidin 9.44%, naringin 29.6%, and quercitrin 90.5%. It can be concluded that under the standard assay conditions, the enzyme is much more active towards quercitrin, indicating a higher specificity for direct linkages from the L-rhamnose residue to the aglycon (Rojas *et al* 2011). *Aspergillus* Rhase activities showing different substrate specificities have been described, but only one Rhase from *A. niger* (Manzanares *et al* 1997) and the two

Table 2.3: Substrate specificity of α -L-rhamnosidases (Yadav *et al* 2010)

S. No.	Organism	Substrate	Type of linkage	Km value (mM)	Specific activity	Reference
1.	*Fagopyrum esculentum*	p-Nitrophenyl-α-L-rhamnoside	-1	0.33	–	Bourbouze *et al* 1976
		6-O-alpha-L-rhamnosyl-D-glucopyranose	-1,4	2.2	–	Bourbouze *et al* 1976
2.	*Bacteroides JY-6*	p-Nitrophenyl- α-L-rhamnoside	-1	0.29	162.57	Jang and Kim 1996
		Neohesperidin	-1,2	0.82	190.21	Jang and Kim 1996
		Naringin	-1,2	0.89	242.67	Jang and Kim 1996
		Poncirin	-1,2	0.93	174.60	Jang and Kim 1996
		Hesperidin	-1,6	1.33	109.31	Jang and Kim 1996
		Rutin	-1,6	1.44	88.12	Jang and Kim 1996
		Saikosaponin C	-1,4	1.6	2.93	Jang and Kim 1996
3	*Pseudomonas paucimobilis FP2001*	Hesperidin	-1,6	0.06	0.12	Miake *et al* 2000
		Proscillaridin A	-1	0.07	1.48	Miake *et al* 2000
		Rutin	-1,6	0.13	0.17	Miake *et al* 2000
		Naringin	-1,2	0.17	0.18	Miake *et al* 2000
		Saikosaponin C	-1,4	0.88	2.52	Miake *et al* 2000
		Quercitrin	-1	0.89	11.20	Miake *et al* 2000
		p-Nitrophenyl-α-L-rhamnoside	-1	1.18	92.40	Miake *et al* 2000
4.	*Fusobacterium K-6*	p-Nitrophenyl-α-L-rhamnoside	-1	0.057	3.40	Park *et al* 2005
		Quercitrin	-1	0.077	5.00	Park *et al* 2005

		Hesperidin	-1,6	0.022	0.52	Park *et al* 2005
		Naringin	-1,2	0.021	0.34	Park *et al* 2005
		Poncirin	-1,2	0.020	0.35	Park *et al* 2005
		Rutin	-1,6	0.028	0.07	Park *et al* 2005
5.	*Clostridium*	p-Nitrophenyl-α-L-rhamnoside	-1	–	82	Zverlov *et al* 2000
	stercorarium	Naringin	-1,2	–	1.5	Zverlov *et al* 2000
		Hesperidin	-1,6	–	0.46	Zverlov *et al* 2000
6.	*Bacillus spGL1 (RhaA*	p-Nitrophenyl-α-L-rhamnoside	-1	0.119, 0.282	104, 79.4	Hashimoto *et al* 2003
	and RhaB)	Naringin	-1,2	–	–	Hashimoto *et al* 2003
		Gellan	-1,3	–	–	Hashimoto *et al* 2003
7.	*Pichia angusta X349*	Naringin	-1,2	–	–	Yanai and Sato 2000
		Rutin	-1,6	–	–	Yanai and Sato 2000
		Hesperidin	-1,6	–	–	Yanai and Sato 2000
		Quercitrin	-1	–	–	Yanai and Sato 2000
8.	*Absidia sp.*	20 (S)-Ginsenoside, 20 (R)-ginsenoside	-1,2, -1,2	–	–	Yu *et al* 2002
9.	*Aspergillus niger*	Naringin	-1,2	1.9	21	Puri and Kalra 2005
10.	*Aspergillus aculeatus* (RhaA and RhaB)	p-Nitrophenyl-α-L-rhamnoside	-1	0.3, 2.8	24, 14	Manzanares *et al* 2001
11.	*Stagonospora avenae*	Avenacoside-α-L-rhamnoside	-1,4	0.091	–	Hughes *et al* 2004

Rhases purified from *A.* (Manzanares *et al* 2000) were reported to be active towards all three of these rhamnoglucosides. The *A. luteo albus* Rhase described in this report displays a broad substrate specificity that allows its application to rhamnoside hydrolysis in industrial processes under alkaline conditions **(Table 2.3)**.

2.11.4 Effect of metal ions and inhibitors

Rhamnosidases tend to undergo changes in their physical and chemical properties in the presence of salts and other substances affecting their catalytic properties. Therefore, it was necessary to test the effects of a number of representative cations and agents in the reaction medium on Rhase activity. Before the assay, the enzyme solution was dialyzed against MB in order to remove all interfering substances. Purified Rhase activity was not affected by β-mercaptoethanol and dithiothreitol. This result suggests there is no critical role of the cysteine residue(s) in the catalysis and/or substrate binding site(s). On the other hand, cations such as Ca^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} did not show any significant effect on Rhase activity under the assay conditions, whereas the enzyme activity was strongly inhibited in the presence of 0.2 mM Zn^{2+}. The chelating agent EDTA also inhibited the enzyme activity, indicating an important role of free cations in the catalysis process and/or in the maintenance of the native three-dimensional structure of the enzyme (Rojas *et al* 2011).

The effects of metal ion on the activity of the purified enzyme of *Penicillium citrinum* MTCC-8897 were determined by Yadav *et al* 2012. The divalent cations Mn^{++} and Ca^{++} enhance the enzyme activity. However, other divalent cations (Cu^{++}, Hg^{++}, Co^{++} and Zn^{++}) and monovalent cations (K^{+} and Na^{+}) inhibit the enzyme activity. Similar results have been reported previously in the literature (Yanai and Sato 2000).

Divalent metal ions such as $Cu^{2+,}$ Fe^{2+}, and Hg^{2+} (1 mM each) were potent inhibitors of the enzyme of *Bacillus* sp. Other divalent metal ions (Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) had no effect on the enzyme activity at 1 mM. Thiol reagents, such as dithiothreitol, glutathione (reduced form), 2-mercaptoethanol, N-ethylmaleimide, iodoacetic acid and a chelator of EDTA (1mM each) revealed no appreciable effect on enzyme activity. L-Rhamnose (the reaction product) considerably inhibited the enzyme activity (70%) at 10 mM, although other saccharides (L-fucose, D-galactose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, Glc, GlcA, D-mannose, L-mannose, and D-xylose) showed no significant effect on enzyme activity (Hashimoto *et al* 1999).

The enzyme from *Aspergillus niger* could be inhibited by Fe^{2+}, Fe^{3+}, Zn^{2+}, Al^{3+}, Mn^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+} ions and SDS (Ni *et al* 2012). Activity was only inhibited by Mg^{2+} (63% inhibition) whereas Ca^{2+} stimulated activity by 14%. DTT showed no effect suggesting that sulphhydryl groups play a minor role, if any, in this enzyme of *Aspergillus nidulans* (Manzanares *et al* 2000).

The presence of 2 mM Co^{2+} had a significant positive effect on the enzymatic activity (Rajal *et al* 2009). Hashimoto and Murata (1998) reported that 1 mM Co^{2+} negatively affected the enzyme from *Sphingomonas sp.* The *Sphingomonas* enzyme was totally inhibited by EDTA. The presence of 2 mM Cu^{2+} showed a strong inhibitory effect (70% reduction of activity). Conversely, the α-rhamnosidase from *A. terreus* (Gallego *et al* 1996) was reportedly not affected at all by this cation. Addition of 2 mM Hg^{2+} resulted in near total inhibition of enzymatic activity. A similar result was reported by Hashimoto *et al* (1999) and Zverlov *et al* (2000) with 1 mM Hg^{2+}. Increased ionic strength did not show a consistent effect on enzyme activity, which varied between 80 and 120% of the initial value (Rajal *et al* 2009).

At 1 mM Cu^{2+} the enzyme activity of *Pseudomonas paucimobilis* FP2001 was almost completely inhibited. Relative activity was lowered to 14% by the addition of Pb^{2+} (0.1 mM), and to 9, 6, and 6% by Cd^{2+}, Zn^{2+} and Ba^{2+} (each 1 mM), respectively. By contrast, the same concentration of Ca^{2+} accelerated the enzyme to a level higher than the control without metal ions. Ca^{2+} chelators such as EDTA and EGTA (1 mM) lowered the enzyme activity to 0.7 and 10%, respectively. The enzyme activities, which had been inhibited by incubation with 1 mM EDTA or EGTA for 10 min at 37 °C, were completely restored by the subsequent addition of Ca^{2+} at 1 mM or 10 mM, respectively, 10 min before assaying the residual enzyme activities. The enzyme activities, which had been inhibited by either 0.1 mM Pb^{2+} or 1 mM Mn^{2+} under the same conditions, were restored to 36% and 76%, respectively, of the control activities by the subsequent addition of 10 mM Ca^{2+}. These results indicated that Ca^{2+} serves as a potential cofactor (Miake *et al* 2000).

2.11.5 Molecular weight of rhamnosidase

The relative molecular masses of the reported α-L-rhamnosidases are in the range 53.0-240.0 kDa though in some cases oligomeric forms of the enzyme having relative molecular mass as high as 500 kDa have been reported (Hashimoto *et al* 2003).

SDS-PAGE revealed an apparent molecular weight of 109 kDa for *A. luteo albus* Rhase (Rojas *et al* 2011). Values ranging from 90 to 96 kDa have been described for *Clostridium stercorarium* (Zverlov *et al* 2000), *Aspergillus terreus* (Gallego *et al* 2001) and two *A. niger* Rhases (Roitner *et al* 1984a) whereas molecular mass values of 70, 72 and 75 kDa were reported for the two N-glycosylated Rhases from *A. aculeatus* (Manzanares *et al* 2000) and another *A. niger* Rhase (Manzanares *et al* 1997), respectively.

The molecular mass of the α-L-rhamnosidase from *Aspergillus kawachii* was 90 kDa (Koseki *et al* 2008) is similar to the molecular mass that has been reported for the extracellular α-L-rhamnosidases from fungi, including *A. nidulans*, 90 kDa (Orejas *et al* 1999) and 102 kDa (Manzanares *et al* 2000); *A. terreus,* 90 kDa (Gallego *et al* 1996); *Penicillium decumbens*, 90 kDa (Young *et al* 1989); *Aspergillus niger*, 85 kDa (Manzanares *et al* 1997); and *A. aculeatus*, 87 kDa (Mutter *et al* 1994) and 92 and 85 kDa (Manzanares *et*

al 2001). The carbohydrate content in the α-L-rhamnosidases from *P. decumbens*, 90 kDa (Young *et al* 1989); *A. niger*, 85 kDa (Manzanares *et al* 1997); *A. nidulans*, 102 kDa (Manzanares *et al* 2000); and *A. aculeatus*, 92 kDa and 85 kDa (Manzanares *et al* 2001) has been reported to be approximately 50%, 12%, 7%, 24%, and 15%, respectively. The α-L-rhamnosidase from *A. kawachii* was observed to be a glycoprotein with a carbohydrate content of approximately 22%. N-linked oligosaccharides are involved in the carbohydrate content and the hyperglycosylation of Ak- RhaA may also be due to high degree of N-glycosylation (Koseki *et al* 2008).

2.11.6 Structure of rhamnosidase

The crystal structure of only one α-L-rhamnosidase, RhaB from the *Bacillus sp.* GL1, is available at 1.9 ´°A resolution (Cui *et al* 2007). The molecular mass of the enzyme is 106 kDa and it contains 956 amino acid residues. RhaB shares the significant structural similarity with chitobiose phosphorylase ChBP from *Vibrio proteolyticus* and maltose phosphorylase MaIP from the *Lactobacillus brevis* though the primary structure of RhaB is significantly different from the primary structure of these two enzymes. The structure of rhamnose bound RhaB has also been determined at 2.1´°A resolution. Rhamnose binds to the deep cleft of (α/α) 6-barrel domain. Several negatively charged residues such as Asp567, Glu572, Asp579 and Glu841 interact with rhamnose and RhaB mutants of these residues drastically reduced the enzyme activity indicating that these residues are crucial for the enzyme catalysis and the substrate binding. There is a scientific need to crystallize α-L-rhamnosidase from other sources and to solve their crystal structures to obtain extensive structural variations in α-L-rhamnosidase (Yadav *et al* 2010).

2.12 Molecular biology of rhamnosidases

Only a few reports on the isolation, cloning and over expression of the gene coding for α-L-rhamnosidase are available (Yadav *et al* 2010). The ram A, which belongs to the new type of glycoside hydrolase family, represents the first cloned α-L-rhamnosidase gene and it was obtained from the anaerobe thermophilic bacterium (Zverlov *et al* 2000). Two genes rhaA and rhaB from *A. aculeatus* encoding the α-L-rhamnosidase RhaA and RhaB respectively were cloned by using polyclonal antibodies (Manzanares *et al* 2003). The genes rhaA and rhaB from *Bacillus sp.* GL1 encoding two different α-L-rhamnosidase were cloned in *E. coli* and over expressed. The RhaA of *Bacillus sp*. GL1 shows 41% sequence identity with RamA of *C. stercorarium*, 23% identity with RhaB of *Bacillus sp*. GL1, while RhaB shows only 20% identity with RamA and 24% identity with RhaA and RhaB of *A. aculeatus*. Only RhaB was produced when *Bacillus sp.* GL1 cell were grown in gellan medium. The gene ramA, rhaA and rhaB are categorised into family GH 78 (Cui *et al* 2007). The cloning and expression of gene rhaM encoding α-L-rhamnosidase of *Sphingomonas paucimibilis* FP2001 was done in *E. coli*. The RhaM protein showed no significant homology to other α-L-

rhamnosidase of glycoside hydrolase family 78 (Miyata *et al* 2005). Mutants of RhaB of *Bacillus sp.* GL1 substituting Asp-567, Glu-572, Asp-579 and Glu-841 with Asn, Gln, Asn and Gln respectively using site directed mutagenesis have been prepared and found to be of reduced enzymatic activity indicating that above amino acids residues are crucial for enzymatic catalysis (Cui *et al* 2007). The two α-L-rhamnosidase genes, rhmA and rhmB were identified in a partially sequenced genome of the bacterium PRI-1686. Whole genes were recovered by amplifying flanking sequences with single specific primers and non-specific walking primers. The recovered genes were then cloned into *E. coli* (Birgisson *et al* 2004). Two putative rhamnosidase genes, ram1LP and ram2LP were identified in the genome of *L. plantarum* and one rhamnosidase gene ramALA was identified in *L. acidophilus* genome (Beekwilder *et al* 2009). The two α-L-rhamnosidase genes rhaB1 and rhaB2 were identified in operon rhaP2B2P1B1, was repressed by glucose and induced by L-rhamnose, showing regulation at transcription level (Avila *et al* 2009). Koseki *et al* 2008 have reported that *A. kawachii* α-L-rhamnosidase encoding gene (Ak-rhaA) encodes for a 655 amino acid protein. The protein possesses 13 potential N-glycosylation recognition sitesand exhibits 75% of sequence identity with the α-L-rhamnosidase belonging to the glycoside hydrolase family 78 from *A. aculeatus* and with hypothetical *Aspergillus oryzae* and *Aspergillus fumigates* proteins. So far no attempt has been made to improve the catalytic efficiency of α-L-rhamnosidase using the technique of directed evolution or site directed mutagenesis (Yadav *et al* 2010).

2.13 Applications of α-L-rhamnosidases

With recent advances, enzyme technology presents an alternative to chemical processes, reducing both energy and material consumption and thus minimizing the generation of waste. In this context, rhamnosidase have been demonstrated to have applications in biotechnology, with a possible foray into industrial uses. These applications are mainly based on the hydrolytic activity of rhamnosidase and are discussed with respect to the chemical, food and pharmaceutical industry (Puri 2012).

2.13.1 Food industry

2.13.1.1 Debittering citrus fruit juices

Bitterness in citrus fruit juices is due to the presence of naringin, which may be removed by treating the juice with naringinase (Puri *et al* 2008, Puri 2012). In this direction, naringinase has been immobilized on various supports for achieving hydrolysis of naringin and eventually debittering citrus fruit juice (Puri and Banerjee 2000; Prakash *et al* 2002; Ferreira *et al* 2008; Ribeiro 2011). The immobilized naringinase on polyvinyl alcohol has been used for the debittering of juices (Busto *et al* 2007). Additionally, the use of purified recombinant enzyme for treating citrus juice, a maiden report where recombinant alpha-L-rhamnosidase has been immobilized in Ca^{2+} alginate beads for extrapolating its hydrolysis in

kinnow fruit juice. This study reports activity and stability of the free and immobilized enzyme (Puri *et al* 2010b). Recently, immobilization of naringinase using mesoporous silica MCM-41 via adsorption with glutaraldehyde has been documented. These supports possess tunable pore size, large surface area, high adsorption capacity, and an ordered porous network for free diffusion of the substrates and reaction products. The immobilized catalyst showed excellent thermal stability and storage stability and could be recycled six times for the treatment of white grapefruit juice (Lei *et al* 2011).

2.13.1.2 Aroma enhancement

Rhamnosidase activity of naringinase in combination with β-glucosidase and arabinosidase was considered suitable for aroma enhancement in wine making. The enzyme was immobilized to a solid carrier with the aim of developing a continuous process for wine aroma enhancement (Gallego *et al* 2000, Puri 2012).

2.13.1.3 Gellan depolymerisation

With respect to the manufacture of food additives, rhamnosidase could be used in the preparation of food additives from biopolymers as well as in the preparation of sweeteners (Giavasis *et al* 2000, Puri 2012). Rhamnosidases play an important natural role in the modification of the viscous property of gellan gum. The α-L-rhamnosidase from *Bacillus sp.* GL1 was used for complete depolymerization of gellan (a heteropolysaccharide to a tetrasaccharide (unsaturated glucuronyl-glucosyl-rhamnosyl-glucose). The enzyme acted on the gellan-degrading product (rhamnosylglucose) formed after successive reactions catalyzed by gellan lyase (Hashimoto and Murata 1998).

2.13.1.4 Tomato pulp digestion

The rhamnosidases from *L. plantarum* have been shown to convert flavonoid rutinosides (such as rutin from tomato) into well absorbed glucosides. Such activity implies that probiotic lactobacilli when present in gut microflora may enhance flavonoid bioavailability (Beekwilder *et al* 2009; Puri 2012).

2.13.2 Chemical industry

2.13.2.1 Enzymatic production of rhamnose

Large amounts of citrus peel (rich in glycosylated poly-phenolic compounds) are generated as a by-product of the juice processing industry. This study facilitated the hydrolysis of naringin extracted from citrus peel waste. The potential of recombinant alpha-L-rhamnosidase in the manufacture of rhamnose from citrus peel was investigated. The result indicated that recombinant L-rhamnosidase has industrial applicability as well as being an interesting candidate for the production of rhamnose and prunin from citrus peel waste (Kaur *et al* 2010, Puri 2012). An efficient method was developed to produce rhamnose by inactivating β-glucosidase expressed by naringinase (Vila-Real *et al* 2010). Naringinase has been used to hydrolyze glucolipids, leading to the synthesis of unique special fatty acids. A

di-rhamnolipid was cleaved by naringinase from *P. decumbens* leading to a mono-rhamnolipid and L-rhamnose (Magario *et al* 2009).

2.13.2.2 Naringin extraction from kinnow peel waste

A carbohydrate containing substrate "naringin" extracted recently from kinnow peel was investigated by the author (Puri 2012). Kinnow (a hybrid between *Citrus deliciosa* and *Citrus nobilis*) peel, a waste rich in glycosylated phenolic substances, is the principal by-product of the citrus fruit processing industry. Recombinant α-L-rhamnosidase purified from *E. coli* cells using immobilized metal chelate affinity chromatography (IMAC) was used for naringin hydrolysis. The purified enzyme was inhibited by Hg^{2+} (1 mM), 4-hydroxymercuribenzoate (0.1 mM) and cyanamide (0.1 mM). The purified enzyme established hydrolysis of naringin extracted from kinnow peel, thus endorses its industrial applicability for producing rhamnose (Puri *et al* 2011). Birgisson *et al* (2004) produced α-L-rhamnosidase from *E. coli* and developed a bioreactor for narigin hydrolysis.

2.13.3 Pharmaceutical industry

2.13.3.1 Deglycosylation of flavonoids

Deglycosylation of flavonoids in *Cleome arabica* leaf extracts (CALE) by naringinase improves the beneficial effect of this plant extract on polymorphonuclear leukocytes (PMNs). The ability of *C. arabica* leaf extract treated with naringinase to reduce chemotaxis in human neutrophils may be an important therapeutic factor in the treatment of chronic diseases (Bouriche and Arnhold 2010, Puri 2012).

2.13.3.2 Preparation of prunin

Prunin possesses anti-inflammatory and antiviral activity against DNA/RNA viruses (Kaul *et al* 1985, Puri 2012). Pure prunin in high yield was obtained from naringin when immobilized naringinase pretreated with alkaline buffer was used for the preparation. The obtained flavonoid prunin has variable antiviral activity against DNA/RNA viruses. The flavonoid prunin possesses anti-inflammatory activity and may be used as sweetening agent in diabetes therapy. The natural flavonone glycoside of naringenin has also been reported to prevent gastric mucosal ulceration in animal models. The studies later observe gastroprotective effect of the glycoside and naringin on ethanol-induced gastric injury. Other rhamnosides acts as cytotoxic rhamnosylated terpenoids, as signal substances in plants or play a role in antigenicity of pathogenic bacteria (Roitner *et al* 1984b). The separation of bittering components (by enzyme rhamnosidase) from the citrus juices was investigated to identify compounds (limonin, naringin, and naringenin) that possess anticarcinogenic benefits. Plant flavonoids may be useful for the treatment of cardiovascular disease as well as associated conditions such as obesity, hepatic steatosis, and type 2 diabetes. Flavonoid naringenin-7-O-glucoside is a potential therapeutic agent for treating or preventing cardiomyopathy associated with doxorubicin (Wood and Bhat 1988; Han *et al* 2008).

2.13.3.3 Prodrug therapy

A drug is released from its prodrug by enzyme action. A system was designed in which drugs of interest are capped with rhamnose and are released by rhamnosidase enzyme. The carbohydrate structure of rhamnosidase was specifically engineered through enzymatic deglycosylation and chemical reglycosylation to activate α-L-rhamnopyranoside to determine its use in prodrug therapy (LEAPT-lectin directed enzyme activated prodrug therapy). Highly localized prodrug activation was achieved, and glycosylation enhanced the rate of uptake of the protein component from the serum, while a reduction in time for potential immunogenic exposure was reported (Robinson *et al* 2004). Prodrugs of doxorubicin and 5-fluorouracil capped by the nonmammalian L-rhamnosyl were released by rhamnosidase to its liver target (Garnier *et al* 2010; Puri 2012).

Chapter- III

MATERIALS AND METHODS

3.1 Isolation of yeast strains

Feta cheese was prepared by inoculating the starter mesophilic cultures (*Lactococcus lactis* sub sp. *lactis* and *Lactococcus lactis* sub sp. *cremoris*; Choozit 230) and thermophilic yoghurt cultures (*Streptococcus thermophilus* and *Lactobacillus delbruckii* sub sp. *bulgaricus*; YO-MIX 532) obtained from Danisco, Niebull, Germany in the milk. The whey so obtained is used for beverage making and yeast strains were isolated from this whey beverage. Appropriate dilutions (10^{-2} to 10^{-4}) were prepared and inoculated on Yeast Extract Dextrose Chloramphenicol (YEDC) agar. The colonies grown on media were randomly selected on the basis of their different morphology and colour characteristics. Selected colonies with different morphology were sub-cultured on modified Glucose Yeast Extract (GYE) agar at 28±2 °C for 48 h to obtain pure cultures. The pure cultures were maintained at 4 °C, until use.

3.2 Screening of yeast isolates for α-L-rhamnosidase production

All yeast isolates were grown on modified GYE medium (g L^{-1}: glucose- 10, peptone- 5, yeast extract- 5 and naringin- 2); pH 6 at a temperature 28±2 °C to induce the development of vegetative morphotypes. Screening of yeast isolates capable of producing naringinase were selected by visual screening on GYE medium containing (0.2%) naringin as inducer. The screened cultures were further checked for their ability to hydrolyze the commercial naringin. The α-L-rhamnosidase activity (RA) was determined using p-nitrophenyl-α-L-rhamnoside (p-NPR, Sigma) as the substrate (Romero *et al* 1985). The reaction mixture consisted of 0.1 mL of 4.8 mM p-NPR solution, plus 0.19 mL of 50 mM sodium acetate/ acetic acid buffer, pH 5.0 and 10 µL of enzyme or buffer (for the blank) and was incubated at 50 °C. Aliquots of 50 µL from the reaction mixture were removed from the growth medium every 2 min and placed into 1.5 mL of 0.5 M NaOH. These aliquots were kept in an ice bath until the absorbance was measured at 400 nm (Rajal *et al* 2009). Standard curve was prepared using 0.02-0.1 µmole mL^{-1} of p-nitrophenol **(Fig. 3.1)**.

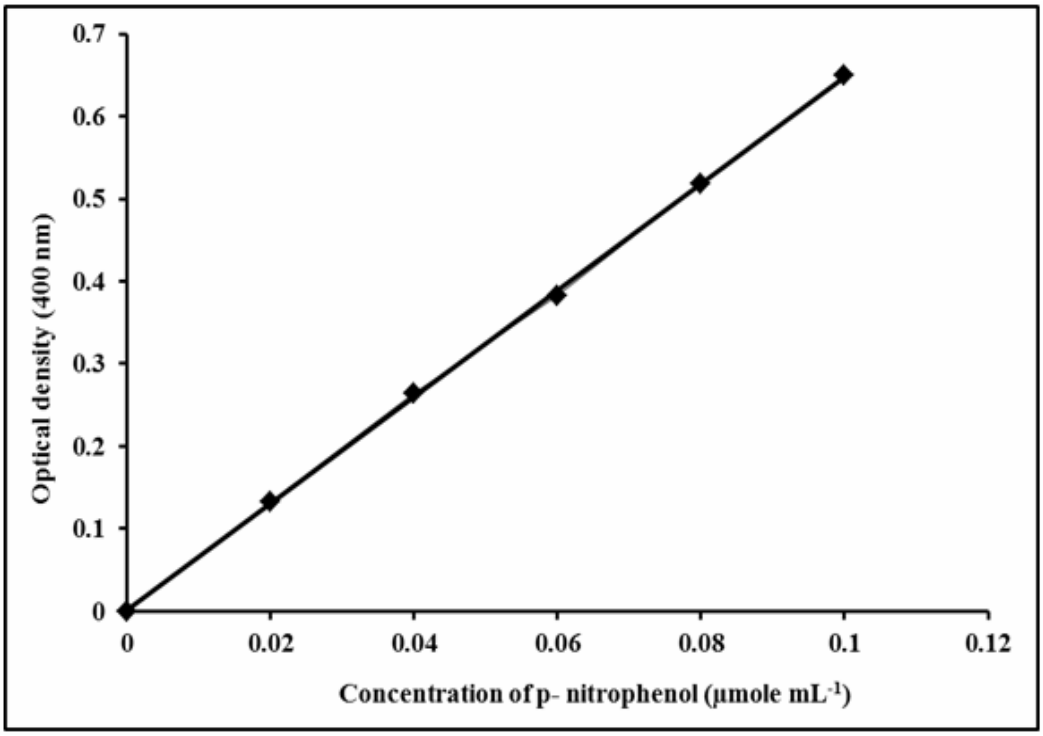


Fig. 3.1 Standard curve of p-nitrophenol

3.3 Characterization of yeast isolates

3.3.1 Morphological characterization

The morphology of yeast cells and their appearance on Glucose Yeast Extract Agar (g L^{-1}: glucose- 10, yeast extract- 5, peptone- 5 and pH- 6) was examined, after incubating at 28±2 °C for 5 days. The following features of the appearance of cultures were recorded: texture, color, shape, pigmentation and surface of colonies (Kreger-van Rij 1984; Kurtzman and Fell 1997). Yeast strains were examined for ascospore formation. The culture was initially incubated for 2 days at 37 °C to facilitate growth, and then further incubated at 28±2 °C to induce ascospore formation. The culture was examined for ascospores at approximately weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation (Kreger-van Rij 1984) carbol-fuchsin and steamed gently for about 5 min. Slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue; the mature ascospores stain red and vegetative cells blue (Kurtzman *et al* 2005).

3.3.2 Biochemical characterization

The identification of the yeast isolates was determined on the basis of biochemical tests including the fermentative growth on specific carbon and nitrogen sources (Van der Walt and Yarrow 1984; Barnett *et al* 2000) as well as other tests like lipase, urea hydrolysis (Van der Walt and Yarrow 1984), acid production from glucose (Kurtzman *et al* 2011), starch, cycloheximide test at 0.01% and 0.1% (Ghosh 2011) and growth on vitamin free medium

(Yarrow 2000) and growth parameters measured at different temperatures ranging from 25-40 °C, pH, incubation time periods and salt concentrations.

3.3.2.1 Fermentation of sugars

The fermentation of D-glucose, D-galactose, D-raffinose, D-xylose and sucrose was tested, according to the method of Van der Walt and Yarrow (1984). A visible suspension of yeast suspended in sterile distilled water was used to inoculate the fermentation medium (g L^{-1}: peptic digest of animal tissue- 10, Sodium chloride- 5, pH- 7.2±0.2) and add desired carbohydrate for checking fermentation pattern in sterile test tubes containing Durham tubes. The tubes were incubated at 28±2 °C on a roll drum for 2 weeks and examined after 3 days, 1 week, and 2 weeks. A positive result was indicated by accumulation of gas in the Durham tubes, as described by Van der Walt and Yarrow (1984). The acidity formed during fermentation can be detected by addition of 1% phenol red indicator, which shows a colour change of the medium from red to yellow under acidic conditions

3.3.2.2 Assimilation of nitrogen compounds

The procedure of Barnett *et al* (2000), involved aliquots (20 mL) of sterile, synthetic basal medium (0.5% ammonium sulphate, 0.1% potassium dihydrogen phosphate, 0.05% magnesium sulphate heptahydrate, 2% purified high grade agar), devoid of a nitrogen source, were melted and cooled to about 40 °C then poured into petri dishes containing about 2 mL suspension of the yeasts under test in sterile peptone water. After solidification, the plates were kept, lid side up, at 28±2 °C for a few hours to obtain a dry surface agar. Small amounts of the various nitrogen compounds were then deposited at different, evenly spaced sites on the agar. Sites were marked on the outside of the smaller dishes. Plates were then incubated at 28±2 °C, lid side down, and results were observed after 3 days. The nitrogen compounds tested were nitrate, nitrite, ethylamine, L-lysine and D-glucosamine. Ammonium sulphate was used as a positive control.

3.3.2.3 Growth on vitamin free medium

The use of the ability or inability to grow in a mineral medium (g L^{-1}: Na_2HPO_4- 6.0, KH_2PO_4- 3.0, NH_4Cl- 1.0, NaCl- 0.5, $MgSO_4$- 0.12, $CaCl_2$- 0.1 and pH- 6) devoid of vitamins was used as a diagnostic property. Actively growing yeast cells from a slant were inoculated into tubes containing mineral medium devoid of vitamins and incubated at 28±2 °C for 7 days. Growth was observed after 7 days. A second vitamin-free tube was inoculated with a standard loop from the first to override growth, which may be due to vitamins carried over from the inoculums.

3.3.2.4 Cycloheximide test

For the determination of cycloheximide resistance, actively growing yeasts were inoculated in the same manner as for the carbon assimilation tests, into tubes containing filter sterilized 0.01% and 0.1% solutions of cycloheximide. Tubes were incubated at 28±2 °C with

agitation for 3 weeks and examined after 3 days, 2 weeks and 3 weeks.

3.3.2.5 Urease test

The urease test was carried out, as described by Van der Walt and Yarrow (1984), using commercial Christensen's urease agar medium (g L^{-1}: peptic digest of animal tissue- 1, dextrose- 1, sodium chloride- 5, disodium phosphate- 1.2, monopotasium phosphate- 0.8, phenol red- 0.012, agar- 18 and pH- 6.8±0.2). Suspend 24.01 g of media in 950 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 10 lbs pressure (115 °C) for 20 min. Cool to 50 °C and aseptically, add 50 mL of sterile 40% urea solution (FD048) and mix well then poured into petri dishes. Actively growing yeast cells were streaked onto agar plates and incubated at 28±2 °C for 24 h. The test was taken to be positive when there was development of a deep red colour in the agar.

3.3.2.6 Starch test

The starch test was carried out by adding 2 drops of Lugol's iodine reagent (5 g iodine and 10 g potassium iodide mixed with 85 mL distilled water, to make a brown solution with a total iodine content of 130 mg mL^{-1}) to the carbon assimilation tubes after 21 days of incubation and noting the colour change.

3.3.2.7 Lipase test

A loopful of culture was spot inoculated into tributyrin agar plates (g L^{-1}: peptic digest of animal tissue- 5, yeast extract, agar- 18 and pH- 7.5±0.2) containing 1 mL of the solvent (white petroleum) and was kept for incubation at 28±2 °C for 24-48 h. Large zone of clearance was observed around the growth on TBA plates after incubation. The formation of large halo zone on TBA plates was observed and assayed for lipase activity.

3.3.3 Monitoring of morphological modification of yeast isolates through scanning electron microscopy

The selected yeast isolates were studied by scanning electron microscopy (SEM) and ascospore surface ornamentation was evaluated. Ascospores were taken after 4 days of incubation and washed in 0.1 M sodium cacodylate buffer (pH 7.4). They were fixed in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4 °C and subjected to (60%, 70%, 80% and 90%) serial alcoholic dehydration treatment (Islam *et al* 2005), followed by post-fixation with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4). Specimens were then dried in a critical point dryer (EMITECH K850, Hitachi) and affixed to SEM stubs using carbon tape followed by thin coating with gold: palladium (60:40) and observed under SEM (Hitachi model S3400 at 15 - 30 kV, 2 -5.00 μm). Yeast strain showing highest rhamnosidase activity was further studied by scanning electron microscopy (SEM) with and without 0.2% naringin.

3.3.4 Metabolic characterization of the yeast isolates

Biolog YT MicroPlate™ system was used to metabolically characterize the rhamnosidase producing yeast. YT MicroPlate technique (Biolog, Hayward, CA, USA), was used to investigate the effect of constant darkness and light on carbon assimilation patterns. Isolates were grown on a special agar Medium (BUY^{TM}) at 28±2 °C and characterized by the Biolog YT MicroPlate system. For each isolates, cells were removed from the agar plate with a sterile swab, and released into a 20 mm diameter tube containing 20 mL of sterile saline solution to reach a final density of 0.5 O.D. at 660 nm. An aliquot of this suspension (150 µL) was inoculated to each well of Biolog YT MicroPlate plate at 28±2 °C for 72 h until a sufficient metabolic pattern is formed (Praphailong *et al* 1997) and the results were registered according to the instructions of the manufacturer. The experiment was performed in triplicate. The reaction data (positive, negative, and borderline responses) from the 24 h readings of the Biolog microplates were recorded for the strains.

3.3.5 Molecular characterization of yeast isolates

Identification of rhamnosidase producing yeast isolates was done primarily by morphological analysis and secondary molecular methods such as DNA extraction, quantification of genomic DNA, PCR amplification of ITS1 and ITS4 regions, DNA sequencing, phylogenetic analysis of the yeast and genetic relatedness based on BOX, ERIC and REP polymerase chain reaction were performed following the standard methods.

3.3.5.1 DNA isolation from yeast cells

Reagents

- **CTAB extraction buffer (2X)**: This was a 2% solution of cetyl trimethyl ammonium bromide (CTAB) in 100 mM Tris.Cl (pH 8.0) which also contained 20 mM of Na_2EDTA (pH 8.0) and 1.4 M NaCl. Immediately before use, β-mercaptoethanol was added to provide a final concentration of 0.1%.
- **TE buffer**: This was a solution of 1 mM Na_2EDTA (pH 8.0) in 10 mM Tris Cl (pH 8.0)
- **Tris-Acetate-EDTA (TAE) 50 X buffer**: This buffer was prepared by dissolving 242 g of Tris base, 57.1 mL of glacial acetic acid and 100 mL of 0.5 M Na_2EDTA (pH 8.0) in distilled water to make one litre.

Procedure

Total DNA from the yeast cells was isolated using a modified CTAB method of Cubero *et al* (1999). Yeast cell pellet from five mL of yeast culture in an Eppendorf tube was suspended in 500 µL of 2X CTAB extraction buffer and incubated at 65 °C for 60 min with intermittent mixing of the tube contents. Thereafter, 500 µL of chloroform-isoamyl alcohol (24:1) was added, the contents mixed by vortexing to form an emulsion followed by centrifugation at 10,000 rpm for 1 min. Using a disposable pipette tip, upper aqueous layer containing DNA was transferred to a clean Eppendorf tube. The DNA was precipitated by

adding 50 µL of 2.5 M sodium acetate (pH 5.2) and 500 µL of isopropanol. The precipitated yeast DNA was collected by centrifugation at 10,000 rpm, 5 min. The DNA in the pellet was washed with cold 70% ethanol and allowed to air dry at room temperature. The air dried DNA pellet was dissolved in 100 µL of TE buffer containing DNAse free pancreatic RNAse A (10 µg mL^{-1}) and stored at -20°C until used.

3.3.5.2 Quantification of genomic DNA

For quality checking and quantification, agarose gel electrophoresis was performed. 5 µL of DNA preparation of all yeast strains along with the standard DNA ladder (Genei) were electrophoresis on 0.8% agarose gel prepared in 1X TBE Buffer. Electrophoresis was carried out at 50 volts for 90 min (Sambrook and Russel 2001). The DNA was stained by ethidium bromide, visualized on UV transilluminator and documented by Bio-Rad digital gel documentation system. The quality was checked from the appearance of the bands. Appearance of sharp bands was considered as good quality DNA preparation. The quantity was determined from the known concentrations of DNA marker as a standard. After quantification, the DNA was diluted by sterile distilled water (Protease, Nuclease free) (GeNeiTM) or TE buffer to the final concentration of about 50 ng μL^{-1}.

3.3.5.3 Amplification of the 18S rRNA gene

The yeast strains producing rhamnosidase enzyme were further identified by sequencing based on partial sequencing ITS1 and ITS4 regions of the rDNA sequence. PCR amplifications of 28S rRNA gene were performed using the specific primers: ITS1 (50-TCCGTAGGTGAACCTGCG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30). PCR reactions were performed in a Peltier Thermal Cycler (BIO-RAD) using 50 ng of total yeast genomic DNA as template. PCR conditions consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of amplification, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Amplified products were resolved by gel electrophoresis (50 V cm^{-1}) on 1.2 % agarose gels (1 X Tris Borate EDTA buffer). A 100 bp ladder (MBI, Fermentas) was used as molecular size marker. The analysis was done at least twice with each enzyme. The fragments were visualized by staining with 1 µL ethidium bromide (10 mg/mL) per ml on agarose gel (1.2 %) and UV illumination. Gels were photographed by Uvitec Gel Documentation system (ALPHA imager).

3.3.5.4 Nucleotide sequencing, accession numbers and phylogenetic tree analysis

PCR products were purified using Quick PCR purification column (Promega, Madison, USA). Purified PCR products were sequenced in ABI3100 Genetic Analyzer with Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, USA) following the manufacturer's protocol. The nucleotide sequences of 18S rRNA gene were deposited in NCBI GenBank. The accession numbers and amplicon size (bp) of 18S rRNA gene nucleotide sequences of the isolates were obtained from NCBI GenBank database**.**

To perform molecular phylogenetic analysis, reference sequences required for comparison were downloaded from the NCBI database. All the sequences of 28S rRNA gene were aligned using the multiple sequence alignment program CLUSTAL W (Larkin *et al* 2007). The aligned sequences were then checked for gaps and saved as molecular evolutionary genetics analysis (MEGA) format in software MEGA v4.0. The pair wise evolutionary distances were computed using the Kimura 2-parameter model (Kimura 1980). To obtain the confidence values, the original data set was resampled 1000 times using the bootstrap analysis method. The bootstrapped data set was used directly for constructing the phylogenetic tree using the MEGA v4.0 program for calculating the multiple distance matrices (Tamura *et al* 2007). The multiple distance matrix obtained was then used to construct phylogenetic trees using neighbour-joining (NJ) method (Saitau and Nei 1987).

3.3.5.5 Genomic DNA fingerprinting using ERIC, BOX and REP PCR

The genomic fingerprints were obtained as described by Rademaker and de Bruijn (1997) to determine phylogenetic relatedness and sequences of different primers. All PCR reactions were carried out in Peltier Thermal Cycler BIORAD. PCR amplifications were performed in a 25 µL reaction volume. Amplified products were electrophoresed on 1.6% agarose gels containing 0.5 µg mL^{-1} ethidium bromide (Sambrook and Russel 2001). A low range ladder (GeNei^{TM}) was used as molecular size marker. The gels were visualized and gel images were documented in BIORAD gel documentation system.

The primers used in the study were Rep1R-I (50-III ICG ICG ICA TCI GGC-30) and Rep 2- I (50-ICG ICT TAT CIG GCC TAC-30), repetitive extragenic palindromic (REP)-PCR; ERIC 1R (50-ATG TAA GCT CCT GGG GAT TCAC-30) and ERIC 2 (50-AAG TAA GTG ACT GGG GTGAGC G-30), enterobacterial repetitive intergenic consensus (ERIC) ERIC-PCR; BOX A1R (50-CTA CGG CAA GGCGAC GCT GAC G-30). The reaction mixture consisted of 1X Gitschier buffer (335 mM Tris-HCl- pH 8.8, 83 mM (NH_4) $2SO_4$, 33.5 mM $MgCl_2$, 33.5 mM EDTA and 150 mM 2-mercaptoethanol), 0.15 mg bovine serum albumin, DMSO (10 %), 2.5 mM total dNTP's, 50 pmol of each primer solution, 3 units of Taq polymerase, 50 ng DNA template and HPLC-grade distilled water. PCR amplification was carried out as described by Versalovic *et al* (1994); Rademaker and de Bruijn (1997) in a Peltier Thermal Cycler (BIO-RAD). The PCR amplification program was performed with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 40 °C (REP-PCR) and 50 °C (ERIC, BOX-PCR) for 1 min and a final extension step at 65 °C for 8 min **(Table 3.1)**.

Table 3.1: PCR conditions for amplification of genetic fingerprinting analysis in this study

PCR conditions	Types of PCR		
A. Reaction mixtures	**BOX**	**ERIC**	**REP**
Taq-/Gitschier-buffer	5 µL	5 µL	5 µL
$MgCl_2$	-	-	-
BSA (20 mg mL^{-1})	0.2 µL	0.2 µL	0.2 µL
DMSO (100 %)	2.5 µL	2.5 µL	2.5 µL
Taq polymerase (3 U)	0.6 µL	0.6 µL	0.6 µL
dNTP mixture (100 mM mix)	1.25 µL	1.25 µL	1.25 µL
Primer (Forward)	1.3 µL (single primer)	1.3 µL	1.3 µL
Primer (Reverse)	-	1.3 µL	1.3 µL
Template DNA	1.2 µL	1.2 µL	1.2 µL
Water (Molecular grade)	12.95	11.65 µL	11.65 µL
B. Reaction conditions			
Lead heat	110°C	110°C	110°C
Initial Temperature	94 °C for 1 min	95 °C for 7 min	95 °C for 5 min
Start cycles	-	-	-
Number of cycles	35	35	35
Denaturations	94 °C for 30 sec	94 °C for 1 min	94 °C for 1 min
Elongation	72 °C for 8 min	65 °C for 1 min	65 °C for 1 min
End cycles	-	-	-
Final extensions	72 °C for 16 min	65 °C for 16 min	65 °C for 16 min

3.3.6 Growth kinetics of yeast in Glucose Yeast Extract (GYE) broth

To study the growth characterstics, a loopful of 24 h old actively growing yeast culture was inoculated in 500 mL Erlenmeyer flasks containing 250 mL GYE broth. It was incubated at 30±5 °C for 24 h to prepare primary inoculum. Primary inoculum was inoculated aseptically in 10 mL and 100 mL GYE broth incubated at 30±5 °C.

3.3.6.1 Optical density

The test tubes containing 10 mL GYE broth were analyzed for growth in terms of increase in optical density at 600 nm using spectrophotometer.

3.3.6.2 Dry weight

The growth was analyzed in terms of increase in cell dry weight; sediments were dried to a constant weight on pre weighed filter paper in the oven at 50 °C and weighed to estimate dry weight, periodically after every 8 upto 72 h.

3.4 Screening of media and juice components for optimization of α-L-rhamnosidase production

The minimal medium (g L^{-1}: glucose- 5.0, Na_2HPO_4- 6.0, KH_2PO_4- 3.0, NH_4Cl- 1.0, NaCl- 0.5, $MgSO_4$- 0.12, $CaCl_2$- 0.1, naringin- 2 and pH- 6) was used for growth and enzyme production. Fifty mL of the resultant medium in Erlenmeyer flask (100 mL) was aerobically cultured at 28±2 °C for 1-4 d on a rotary shaker at 150 rpm. After centrifugation (12,000 × g, 4 °C, for 15 min), the supernatant was collected to measure rhamnosidase activity as described above (Romero *et al* 1985). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute.

The media composition was optimized following 'one-at-a-time' approach to increase α-L-rhamnosidase production. Six different carbon sources (glucose, lactose, sucrose, glycerol, fructose and rhamnose) were added individually at 5 g L^{-1} in the minimal medium containing 0.2% naringin. Four organic nitrogen sources (1g L^{-1}: - peptone, yeast extract, casein and urea) and two inorganic nitrogen sources (1g L^{-1}: ammonium chloride and ammonium sulphate) were also tested individually keeping another factor constant. The effect of temperature in a range between 15 to 45 °C and pH in a range of 3 to 8 on enzyme activity was examined. Further, best carbon and nitrogen supplementation were used at different concentrations from 0.1 to 1%. For each parameter optimization, three sets of independent experiments were carried out and the average value was reported (Chen *et al* 2010; Singh *et al* 2012).

The effect of percent yeast inoculum concentration (v/v- 0.25, 0.5, 0.75, 1 and 1.25), total soluble solids (°B- 12, 13, 14, 15 and 16), temperature (°C- 15, 20, 25, 30 and 35) and time (h- 12, 24, 36, 48 and 60) on α-L- rhamnosidase enzyme production in kinnow juice was also evaluated. Diluted kinnow juice (50 mL) in Erlenmeyer flask (100 mL) was aerobically cultured at 30±5 °C for 48 h on a rotary shaker (150 rpm). After centrifugation (12,000 × g for 10 min), the supernatant was collected for measurement of rhamnosidase activity as described above (Romero *et al* 1985).

3.5 Experimental design

The statistical analysis of the results from experiment 3.4 was performed with the aid of "Design-Expert-9.0.3" (Stat Ease, Inc., Minneapolis, USA). A 25 factorial central composite experimental design, with four factors and five replicates at the centre point, leading to a set of 30 experiments, was used to optimize the production of rhamnosidase from yeast strain 84. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated, and the full experimental plan with respect to their values in actual and coded form, are listed in **Table 3.2**. Upon completion of the experiments, the average maximum rhamnosidase yield was taken as the dependent variable or response (Y). A second-order polynomial equation was then fitted to the data by

Table 3.2: Variables representing medium components used in response surface methodology

Design Summary										
File Version			9.0.3.1							
Study Type	Response Surface		Runs		30					
Design Type	Central Composite		Blocks		2					
Design Model	Quadratic		Build Time (ms)		78					
Factor	**Name**	**Units**	**Type**	**Subtype**	**Minimum**	**Maximum**	**Coded**	**Values**	**Mean**	**Std. Dev.**
A	Rhamnose	G	Numeric	Continuous	-0.15	0.85	-1.000=0.1	1.000=0.6	0.35	0.227429413
B	Yeast extract	G	Numeric	Continuous	-0.15	0.85	-1.000=0.1	1.000=0.6	0.35	0.227429413
C	pH	-	Numeric	Continuous	2	6	-1.000=3	1.000=5	4	0.909717652
D	Temperature	°C	Numeric	Continuous	25	45	-1.000=30	1.000=40	35	4.548588261

the multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC$$

$$Y = + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

$$Y = + \beta_{11} A_2 + \beta_{22} B_2 + \beta_{33} C_2 + \beta_{44} D_2$$

Where: A= rhamnose, B= yeast extract, C= pH, D= incubation temperature (°C), Y= predicted response, β_0= intercept; β_1, β_2, β_3 and β_4= linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}= interaction coefficients and β_{11}, β_{22}, β_{33} and β_{44}= squared coefficients.

Analysis of variance (ANOVA) was performed. The proportion of variance explained by the polynomial models obtained was given by the multiple coefficient of determination (R_2). In order to confirm the maximum rhamnosidase production predicted by the model, three-dimensional response surface and contour presentations were plotted to find the concentration of each factor for maximum rhamnosidase production. The response surface curves were plotted for the variation in rhamnosidase yield as a function of the concentrations of one variable when all the other factors were kept at their central levels. The optimum concentration of each nutrient was identified based on the peak in the three dimensional plot.

3.6 Production, purification and characterization of α-L-rhamnosidase enzyme from yeast strain 84

3.6.1 Production of α-L-rhamnosidase

The α-L-rhamnosidase enzyme production by yeast strain 84 was carried out in a 5 L flask containing 2 L of optimized media composition from experiment 3.4 (g L^{-1}: rhamnose- 6.0, Na_2HPO_4- 6.0, KH_2PO_4- 3.0, yeast extract- 4.0, NaCl- 0.5, $MgSO_4$- 0.12, $CaCl_2$- 0.1, naringin- 2 and pH 4) which was inoculated with 0.75% (v/v) of culture and incubated at 40 °C with an agitation of 150 rpm. Two-day-old culture was taken and centrifuged at 12,000 rpm for 10 minutes at 4 °C. The supernatant was collected and used for determination of rhamnosidase activities as described above.

3.6.2 Ammonium sulphate precipitation

The supernatant was subjected to precipitation at 0-30%, 30-60% and 60-90% ammonium sulphate saturations (Scheme 1) as per protocol (Scopes 1994). The precipitated protein was collected by centrifugation at 12,000 rpm for 10 min at 4 °C. The precipitate was dissolved in 0.1 M sodium acetate / acetic acid buffer pH 5.0 and was dialyzed against 0.01 M sodium acetate / acetic acid buffer of pH 5.0 using 1:1000 excess of the buffer with three changes in 24 h. The activity of rhamnosidase was tested in the protein dialysate and was further purified by DEAE-sephadex column chromatography.

Plate 3.1 (A) Dialysis bag containing precipitated protein

(B) Enzyme purification by DEAE-sephadex column chromatography

3.6.3 Enzyme purification by DEAE-sephadex column chromatography

3.6.3.1 Activation of DEAE-sephadex

Excess of 0.5 N HCl was added to about 30 g of DEAE-sephadex, mixed thoroughly, kept for 30 min and supernatant was removed slowly. This procedure was repeated five times followed by washings with distilled water 3 times. Then sufficient amount of 0.5 N NaOH was added to the above treated DEAE-sephadex in the same way five times and washed with distilled water so that maximum DEAE-sephadex had settled down and its colour became white from creamish. Before packing into the column of height 45 cm and diameter of 3.0 cm (procured from Borosil), DEAE-sephadax was given washings with 0.1 M sodium acetate / acetic acid buffer (pH 5.0). The slurry of activated DEAE-sephadex was poured in the column without interruption. The column was allowed to settle down (length of about 30 cm) uniformly and was equilibrated with 0.1 M sodium acetate / acetic acid buffer (pH 5.0) until the eluate gave pH.

3.6.3.2 Purification of Enzyme

The dialyzed protein (15 mL) was used as analyte for the anion exchange column (DEAE-sephadex) equilibrated with 0.01 M sodium acetate / acetic acid buffer of pH 5.0. The column was washed with the same buffer and the elution of protein was done by varying concentration of NaCl (0.1-1.0 M) in the same buffer (50 mL of the buffer + 50 mL buffer containing 1 M NaCl). The fractions of 3 mL min^{-1} was eluted and analyzed for rhamnosidase activity (Romero *et al* 1985) as described above and protein concentration by Lowry's method (Lowry *et al* 1951). The fractions showed higher rhamnosidase activity were combined and concentrated by putting in dialysis bag, which was kept in solid powdered sucrose at 4 °C **(Plate 3.1)**. The purified, concentrated enzyme sample (15 mL) was lyophilized and stored in the refrigerator for further experiment (Yadav *et al* 2010).

Estimation of protein by Lowry method (Lowry *et al* 1951)

Reagents:

Reagent 1:

Lowry A: 2.0 % Sodium carbonate in 0.1 N Sodium hydroxide.

Lowry B: 1.0 % Copper sulphate.$5H_2O$ in 2% Potassium sodium tartarate.

Reagent 1 was prepared immediately before use by mixing Lowry A and Lowry B reagent in the ratio of 50:1.

Reagent 2: This was 1 N solution prepared by dilution of commercial 2 N Folin Ciocaleu reagent (Sisco research Chemicals) with water to prepare the color-devolping reagent.

Procedure: The sample (0.5 mL) was mixed with 5 mL of reagent 1. After 10 minutes at room temperature, 0.5 mL of reagent 2 was added. The contents were mixed thoroughly and kept at room temperature for 30 min. The absorbance was read at 520 nm using VIS Double Beam Spectro 1203 (Systronics). The standard curve was prepared using 20-100 µg mL^{-1} of

BSA (Bovine Serum Albumin) **(Fig. 3.2)**. The amount of amino acids released in the reaction mixtures was analyzed and calculated by comparing OD_{520} values with the standard curve.

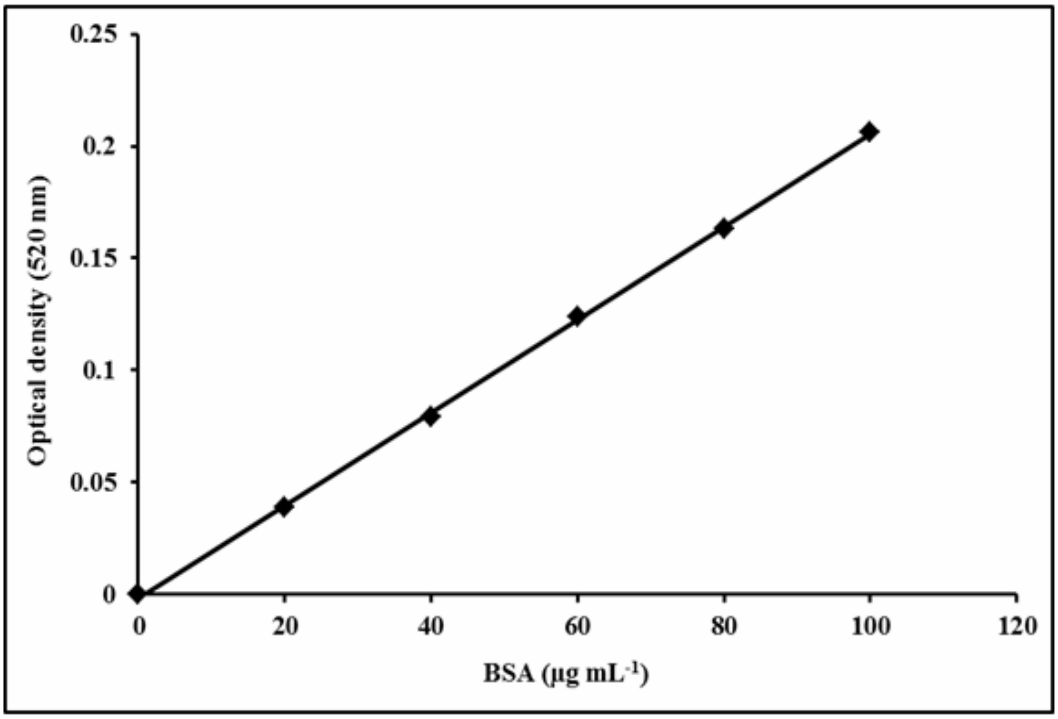


Fig. 3.2 Standard curve of BSA

3.7 Enzyme characterization

3.7.1 Determination of optimum pH

The pH optimum of the purified enzyme was determined using p-nitrophenyl-α-L-rhamnopyranoside as the substrate and measuring the steady-state velocity of the enzyme catalysed reaction in solutions with pH in the range 2 to 8. The buffers used were 0.5 M NaH_2PO_4 /NaOH. The steady-state velocity was plotted against pH of the reaction solutions and pH optimum was calculated from the graph (Yadav *et al* 2012).

3.7.2 Determination of optimum temperature

The temperature optimum was determined by measuring the steady-state velocity of the enzyme catalyzed reaction in solutions of varying temperatures (20-80 °C) using p-nitrophenyl-α-l-rhamnopyranoside as the substrate. The steady-state velocity of the enzyme catalyzed reaction was plotted against the temperature of the reaction solution and temperature optimum was calculated from the graph (Yadav *et al* 2012).

3.7.3 Thermal stability of enzyme

The thermal stability was determined by incubating the aliquots of the enzyme at different temperatures (20, 30, 40, 50, 60 and 70 °C) at the interval of 30 min for 2 h. The enzyme activity was plotted against the time for which the enzyme was exposed at that temperature.

3.7.4 Effect of metal ions on rhamnosidase activity

The effects of various metal ions (Mn^{++}, Cu^{++}, Ca^{++}, Zn^{++}, Co^{++}, Mg^{++}, K^{+}, Na^{+} and Ag^{+}) on rhamnosidase activity were investigated by adding 1 mM of the ions in the reaction solution and determining the activity.

3.7.5 Determination of Km and Vmax

The Michaelis-Menten behaviour of the purified enzyme for the substrate p-nitrophenyl-α-L- rhamnopyranoside was determined by measuring the steady-state velocity of the enzyme catalysed reaction at different concentrations of p-nitrophenyl-α-L-rhamnopyranoside (0.05-1.5 mm) using the reported method (Romero *et al* 1985). The Km and Vmax values were determined by linear regression analysis of data points of the double reciprocal plots (Yadav *et al* 2012).

3.8 Analytical methods

Molecular weight of the purified rhamnosidase was determined by SDS-PAGE (10%) according to Laemmli 1970 and the proteins separated were stained with Coomassie Brilliant Blue R-250.

3.8.1 Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS- PAGE)

The SDS- PAGE analysis of proteins involved electrophoretic separation of individual protein components in a sample in a 11% polyacrylamide gel (1 mm thick) sandwitched between two glass plates (20×20 cm). The gel with separated proteins is stained and the size of particular protein band determined using a co-migrating protein ladder (20 to 80 kDa BioLit™, Sisco Research Laboratories. It involved the following steps:

- Preparation of SDS-polyacrylamide gel plates
- Preparation of samples
- Loading of samples and electrophoresis
- Staining of gel.

3.8.1.1 Preparation of SDS-polyacrylamide gel plates

Solutions required for pouring gel

- **Buffer A (0.20% SDS in 0.75 M Tris.Cl, pH 8.8)**: 90.8 g of Tris Base was dissolved in 900 mL of distilled water, pH adjusted to 8.8 with conc. HCl and volume made to one litre with distilled water.
- **Buffer B (0.20% SDS in 0.25 M Tris.Cl, pH 6.8)**: 10.28 g of Tris Base was dissolved in 900 mL of distilled water, pH adjusted to 6.8 with conc. HCl and volume made to one litre with distilled water.

Both the buffers were sterilized by autoclaving, cooled to room temperature and 2.0 g (per litre) of sodium dodecyl sulphate was dissolved into these buffers.

- **Acrylamide Mix: (44% acrylamide in 0.8% Bis-acrylamide)**: A total of 220 g of electrophoresis grade acrylamide and 4 g N-N methyl bis acrylamide were dissolved in distilled water to make 500 mL. The acrylamide solution was filtered through sintered glass funnel (G-1) and stored in a brown bottle in dark.

- **Ammonium Persulphate solution (10%)**: A total of 100 mg of Ammonium Persulphate was dissolved in one mL of distilled water (Fresh preparation).

- **Running buffer**: This buffer was prepared by dissolving 3.02 g of Tris base, 18.8 g of Glycine and 1.0 g of SDS in distilled water to make one litre.

- **Protein loading buffer**: This solution was prepared by dissolving 2 g of SDS, 1 g of bromophenol blue, 10 mL glycerol in 100 mL of 50 mM Tris Cl (pH 6.8). The solution was optionally mixed with dithiothreitol to provide 100 mM concentration and stored at 4°C.

- **Analytical gel solution (11%)**: Buffer A 24.7 mL was mixed with 12.4 mL of 'acrylamide mix' and 11.0 mL of distilled water. To this solution, 174 µL of ammonium persulphate (10% solution) and 152 µL of TEMED (N,N,N',N'-tetramethylethylene diamine) were added immediately before filling 1 mm space between the two clamped plates of PAGE electrophoresis system.

- **Stacking gel solution (5%)**: Buffer A 15 mL was mixed with 3.75 mL of Acrylamide mix and 12.0 mL of distilled water. To this solution, 75 µL of ammonium persulphate (10% solution) and 60 µL of TEMED were added immediately before layering over the solidified analytical gel in the PAGE electrophoresis system.

- **Gel staining solution**: A 0.25% solution of Commassie blue dye (R-250) in 'gel destaining solution' which consisted of methanol: acetic acid: water: 45:9:46.

Procedure for gel preparation

Casting of SDS-PAGE gel plates

The SDS-PAGE gels consisted of two layers- the lower analytical gel (11%) and upper resolving gel (5%). Analytical gel solution was filled into 1 mm space between the two clamped plates of PAGE electrophoresis system that were held on the casting base, until 3 cm from the top. The gel surface was covered with a thin layer of isopropanol and gel allowed to photo-polymerize at room temperature for 30 min. Thereafter, isopropanol layer was removed by tilting and gel surface washed with distilled water. Stacking gel solution was poured till top, a 16 well comb inserted into the stacking gel and gel allowed to polymerize at room temperature for 30 min. Thereafter, the gel assembly was fitted into PAGE electrophoresis tank and tank filled with the running buffer to appropriate level. The comb was removed to form vertical wells for loading of protein samples.

3.8.1.2 Sample preparation and loading into SDS-PAGE gel

Protein solution 50 µL (purified rhamnosidase enzyme) was suspended into 100 µL of protein loading buffer, the mixture held in boiling water for 3 min and stored in refrigerator. Before use, the samples so processed were centrifuged (12,000 rpm, 3 min) and appropriate volumes (5-25 µL) of the supernatant (to provide equal amount of protein) loaded into individual wells of the gel. An appropriate protein molecular weight standard (10 to 100 kDa) was also loaded in one of the side lanes

3.8.1.3 Running the gel

The PAGE electrophoresis system after loading with different protein samples was connected with power supply and allowed to run constant current (~35 mA in stacking gel and ~50 mA in the analytical gel) till the blue dye front had reached around one cm from the lower edge of the gel.

3.8.1.4 Staining the gel

Remove the gel plate assembly with sandwiched gel from the electrophoresis apparatus and separate the plates with the help of spatula. The gel was carefully removed from the plate and immersed in 'Gel staining solution' in a tray that was kept on a rotary shaker (10 rpm) for 30 min. Thereafter, the staining solution was replaced with destaining solution to remove excess dye from the gel in order to make the separated protein bands clear. The pattern of protein bands was recorded on white light transilluminator of a gel documentation system and observed for proteins of desired size in comparison with mobility of individual proteins in the co-run molecular weight marker (10 to 100 kDa).

3.9 Test for food grade enzyme

3.9.1 Investigation of the oral acute toxicity of the concentrated purified rhamnosidase enzyme

The oral acute toxicity of the purified enzyme was investigated in sweet albino male mice according to Oyedeji *et al* (2013) and Ni *et al* (2012). The mice were fed in cages for 7 days prior to oral administration for their acclimatization to the laboratory conditions. Three adult male mice (age- 6 months, weight 20-25 g) were divided into three groups (Group I- 0.5 mg/g BW and Group II- 1 mg/g BW of enzyme and Group III- 0.5 mL distilled water as the control). In addition, prior to the oral administration, all mice were subjected to a short fasting period of 4 h. The animals were observed for 10 days for behavior changes, symptoms of toxicity and mortality.

3.9.2 Determination of heavy metal ions in concentrated purified rhamnosidase enzyme

Heavy metal ions were determined by an inductively coupled plasma-mass spectrometer (ICP-MS). The purified enzyme sample was analyzed in Department of Soil Science, Punjab Agricultural University, Ludhiana, Punjab, India.

3.10 Debittering of kinnow juice

3.10.1 Selection of Fruit

Kinnow (*Citrus reticulata* Blanco) was procured from Department of Fruit Science, PAU, Ludhiana, Punjab, India. Healthy fruit were selected after manually sorting and discarding defective fruit.

3.10.2 Extraction of juice

Kinnow fruit was washed in chlorinated water and then cut into two pieces. Juice was extracted by kinnow juice extractor under hygienic conditions. Extracted juice was filtered through muslin cloth.

3.10.3 Inoculum preparation

The inoculum was prepared in diluted juice with brix adjusted to (13 °B). A loopful culture of 24 h old yeast (*Clavispora lusitaniae* 84) was inoculated in 100 mL diluted kinnow juice in 250 mL Erlenmeyer flask and incubated at 28±2 °C for 24 h to achieve concentration of 10^5- 10^6 cells mL^{-1}.

3.10.4 Preparation of sugar solution

Granulated sucrose was procured from local market. The sugar solution was prepared by boiling (500 g) granulated sucrose in one litre of water for 10 min and then allowed to cool at room temperature and stored aseptically in sterlized glass bottles.

3.10.5 Physico-chemical analysis of kinnow juice

The physico-chemical analysis: TSS (°B), pH, acidity (% citric acid), Brix acid ratio, total sugars, reducing sugars, ascorbic acid, total phenols and juice yield of kinnow juice was done. Juice was diluted in the ratio 1:1.5 with water. Diluted juice was pasteurized at 82 °C for 15 sec, cooled and brix adjusted to 13 °B by adding sugar solution.

3.10.6 Preparation of debittered kinnow juice

The concentration and incubation time of purified α-L- rhamnosidase enzyme in debittering of kinnow juice was optimized. Debittering of kinnow juice was started by adding different concentration of purified enzyme (control- 0 IU mL^{-1}, T1- 0.2 IU mL^{-1}, T2- 0.4 IU mL^{-1}, T3- 0.6 IU mL^{-1}, T4- 0.8 IU mL^{-1} and T5- 1 IU mL^{-1}) into diluted kinnow juice (13 °B) and kept for 10 h at room temperature. Samples were taken after 2 h of interval for estimation of naringin by Davis method (Davis 1947) as described in method 3.10.9.1.10. The decrease in the naringin content was directly related with reduction in bitterness and optimized conditions were used for large scale production of debittered kinnow juice.

3.10.7 Preparation of debittered kinnow beverage

A debittered kinnow beverage was prepared under optimized conditions of inoculum concentration- (0.75% v/v), TSS (13 °B), incubation temperature (30±5 °C) and incubation time (48 h) from experiment 3.4.

The diluted juice was inoculated @ 0.75% v/v with freshly prepared inoculum and

incubated at 30±5 °C for 48 h in batch scale glass digester.

3.10.8 Bottling and Storage

The beverage was refrigerated for 24 h, siphoned, bottled and then stored in refrigerated conditions (4 °C).

The beverage bottles can also be stored at room temperature after the pasteurization at 72 °C for 15 sec.

3.10.9 Quality evaluation and shelf life determination of kinnow beverage

Shelf life of fermented debittered kinnow beverages, stored at refrigerated (4 °C) and room temperature was studied and evaluated fortnightly for physicochemical, microbiological and sensory qualities.

Mineral analysis of kinnow beverage before and after fermentation was analyzed in Department of Soil Science, Punjab Agricultural University, Ludhiana, Punjab, India.

3.10.9.1 Physicochemical Analysis of Juice

3.10.9.1.1 pH

pH of the juice was determined using a digital pH meter (Electronic Corporarion of India Ltd., Hyderabad, type 101).

3.10.9.1.2 Total Soluble Solids

Total soluble solids (TSS) in juice and beverage were determined by using Erma hand refractometer of 0-32 °B (UNICO make). A drop of distilled water at 20 °C was placed on clean and dry prism and calibration was done at zero line on the scale. Then the samples of juice and beverage were analyzed for their TSS value by reading the line of demarcation on the scale.

3.10.9.1.3 Titrable Acidity

Total acidity expressed as citric acid was estimated following the procedure of AOAC (1999). Titrable acidity was determined by titrating known quantity of water extract of fresh fruit (10mL) against standardized 0.1N NaOH using a few drops of 1% phenolphthalein solution as indicator to pink end point which should persist 15 sec. Results were expressed as % anhydrous citric acid for fresh fruit.

$$\text{Acidity (\% citric acid)} = \frac{\text{Titre} \times \text{Normality of alkali used} \times \text{Volume made} \times \text{Equivalent weight}}{\text{Wt. of sample} \times \text{Aliquot used} \times 1000} \times 100$$

3.10.9.1.4 Brix-Acid Ratio

Brix - acid ratio was calculated by dividing TSS value with total acidity of the juice and carbonated beverage, to determine the ripeness of fruit.

3.10.9.1.5 Total sugars

Total sugars were estimated by phenol-sulphuric acid method of Dubois *et al* (1956) using glucose as standard. For estimation purposes, aliquots of 0.1-0.5 mL sugar solution/beverage were taken in test tubes and distilled water was added to make the volume 1

mL. It was followed by addition of 1 mL 5% phenol and 5 mL conc. sulphuric acid. The acid was poured directly in the centre of the test tube to ensure that temperature rises to 70 °C for optimal color development. The test tubes were kept for 10 min at room temperature and then cooled under tap water. A stable yellow orange color developed after about 20 min. Absorbance was recorded at 490 nm using VIS Double Beam Spectro 1203 (Systronics) against a reagent blank. The concentration of total sugars was calculated from the standard curve by using glucose (20-100 µg mL^{-1}) **(Fig. 3.3).**

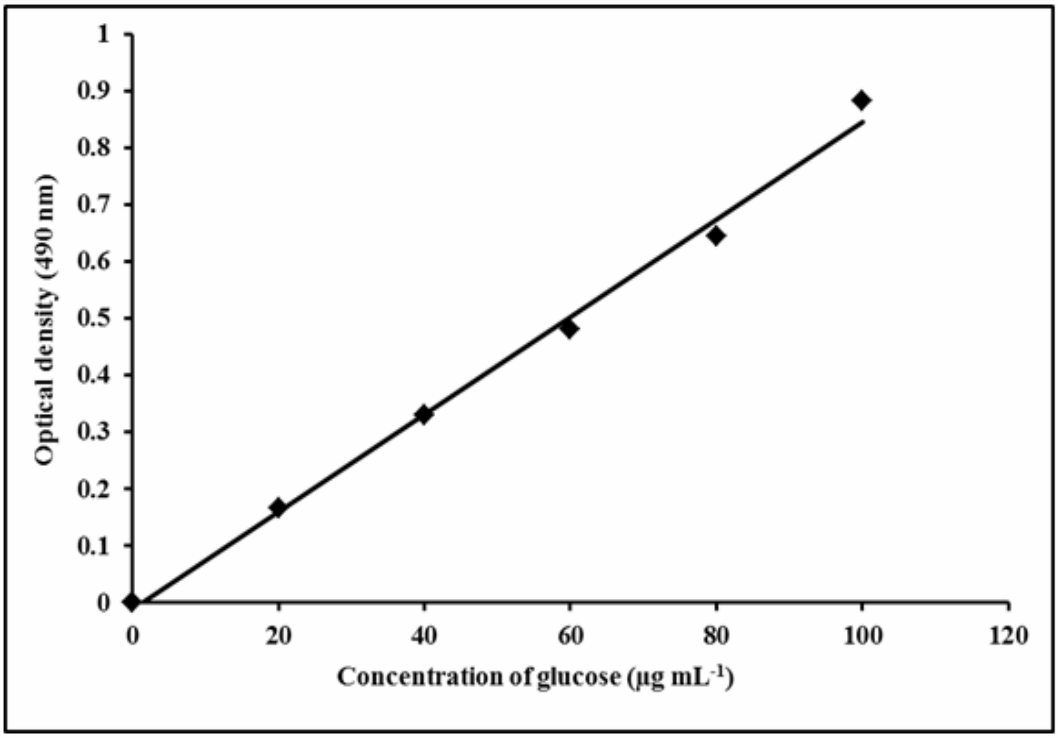


Fig. 3.3 Standard curve of glucose

3.10.9.1.6 Reducing sugars

Reducing sugars were estimated by the method of Miller (1959). Test tubes containing 3 mL sample and 3 mL DNS reagent (10 g of 3, 5 dinitrosalicyclic acid, 2.0 g phenol and 0.5 g sodium sulphite solution dissolved in 500 mL 1% sodium hydroxide solution and the volume was made 1000 mL by adding additional alkaline solution, filtered and stored in a dark colored bottle) were heated for 15 min in a boiling water bath. 1 mL Rochelle salt solution (40 g sodium potassium tartarate was dissolved in distilled water and the volume was made to 100 mL) was added to each tube and the tubes were allowed to cool to room temperature. O.D. was measured at 575 nm using spectrophotometer.

The concentration of reducing sugars was calculated from the standard curve by using glucose (20-100 µg mL^{-1}) **(Fig. 3.4).**

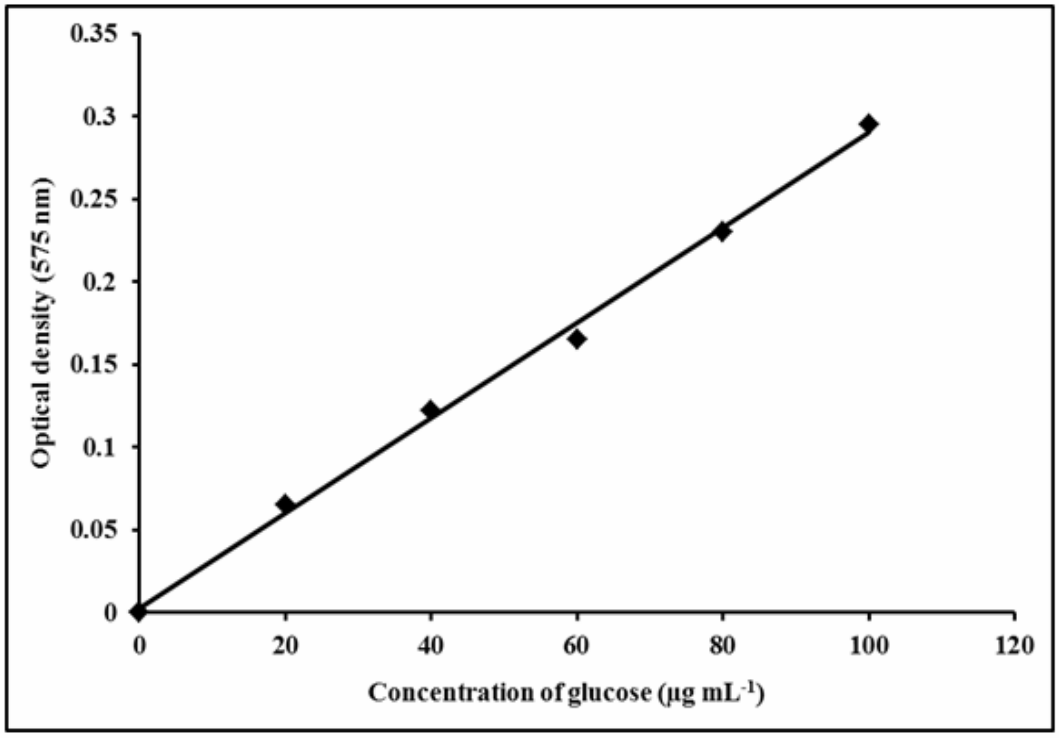


Fig. 3.4 Standard curve of glucose

3.10.9.1.7 Ascorbic acid

The titrametric method using 2, 6-dichlorophenol indophenol dye was used to estimate ascorbic acid (AOVC 1996). Dye factor (i.e. mg of ascorbic acid per mL of dye) was determined by 5mL standard ascorbic acid solution and 5mL 0.4% oxalic acid against dye solution to a pink colour. Known weight of crushed sample (10 g) or known volume of beverage (10 mL) was taken and 100 mL of volume was made up with 0.4% oxalic acid solution. The mixture was filtered through Whatman filter paper no. 4. To a measured volume of aliquot (10 mL), 15 mL of oxalic acid (0.4%) was added followed by titration against standardized dye (0.04%) to a pink end point which should persist for at least 15 sec. Fresh dye solution and standardized ascorbic acid was prepared before each analysis.

$$\text{mg of ascorbic acid/100 g} = \frac{\text{Titre} \times \text{Dye factor} \times \text{Volume made}}{\text{Aliquot taken} \times \text{Weight of sample}} \times 100$$

3.10.9.1.8 Total polyphenol assay

The total phenolic content (TPC) was determined by spectrophotometry, using gallic acid as a standard, according the method described by Singleton and Rossi (1965). Briefly, 0.2 mL of the diluted sample extract was transferred in tubes containing 1.0 mL of a 1/10 dilution of Folin-Ciocalteu's reagent in water. After waiting for 10 minutes, 0.8 mL of a sodium carbonate solution (7.5% w/v) was added to the sample. The tubes were then allowed to stand at room temperature for 30 min before absorbance at 743 nm was measured. The TPC was expressed as gallic acid equivalents (GAE) in mg/100 mL of fruit juice. The

concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 0.5 to 2.5 mg L^{-1} (**Fig. 3.5**).

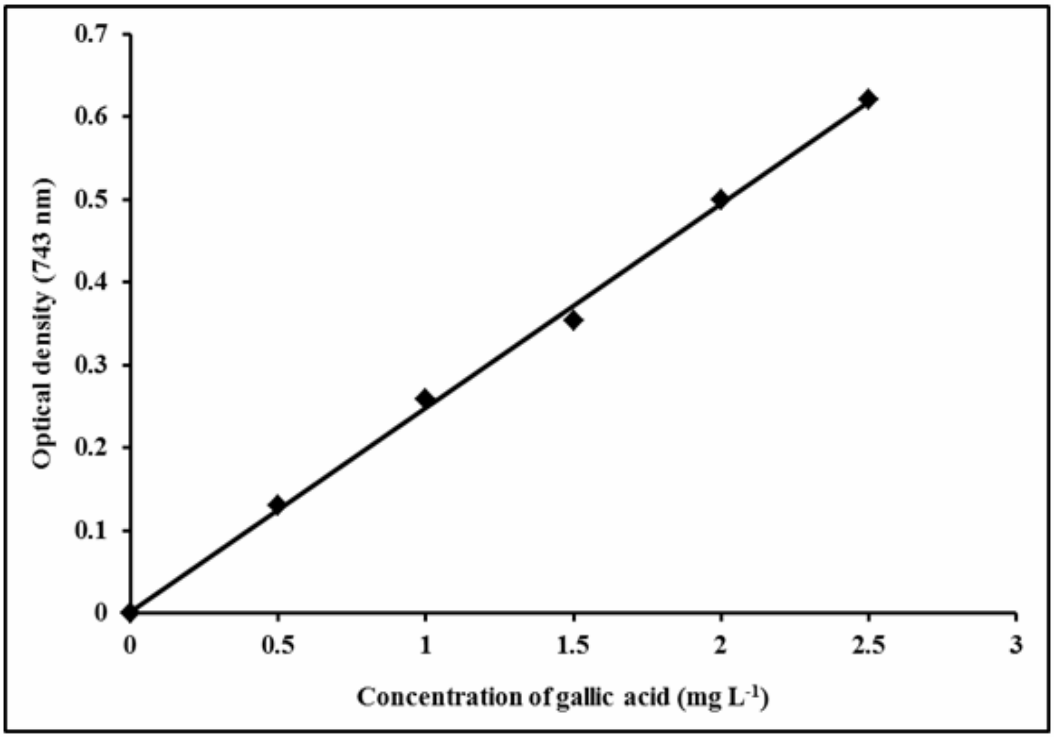


Fig. 3.5 Standard curve of gallic acid

3.10.9.1.9 Limonin estimation

The limonin was estimated from the chloroform extract of sample by colorimetric method (Vaks and Lifshitz 1981). Five mL of centrifuged juice, made to 25 mL with distilled water was extracted with petroleum ether (b.p.60-80 °C) in a separatory funnel (250 mL) to extract the coloring matter. The petroleum ether extract was discarded and aqueous solution was extracted with chloroform (3x25 mL). The chloroform extract was washed with distilled water (4x50 mL). The volume was made to 50 mL with chloroform. A known quantity of this solution was used for determination of d-limonin by developing color with Burham's reagent (0.1 g of 4-dimethyl amino benzaldehyde dissolved in 3 mL of glacial acetic acid and mixed with 2.4 mL of 70% perchloric acid), followed by stirring vigorously on an electric stirrer. The Burham's solution was made fresh prior in every estimations.

Preparation of d-limonin standard

Standard solution of d-limonin was prepared by dissolving 1.0 mg of d-limonin in chloroform and volume was made to 100 mL. Different volumes i.e. 1, 2, 3, 4 and 5 mL of chloroform solution containing limonin concentrations 10, 20, 30, 40 and 50 µg respectively were taken in separate test tubes along with blank. The tubes containing chloroform with different concentrations of standard were heated in a water bath for 10 min and cooled. To the

residue of each test tube, added 3 mL of Burham's reagent, after 30 min the intensity of red color so developed was measured with VIS Double Beam Spectro 1203 (Systronics). The standard curve was plotted between different concentrations of d-limonin and the corresponding optical densities (503 nm) **(Fig. 3.6)**.

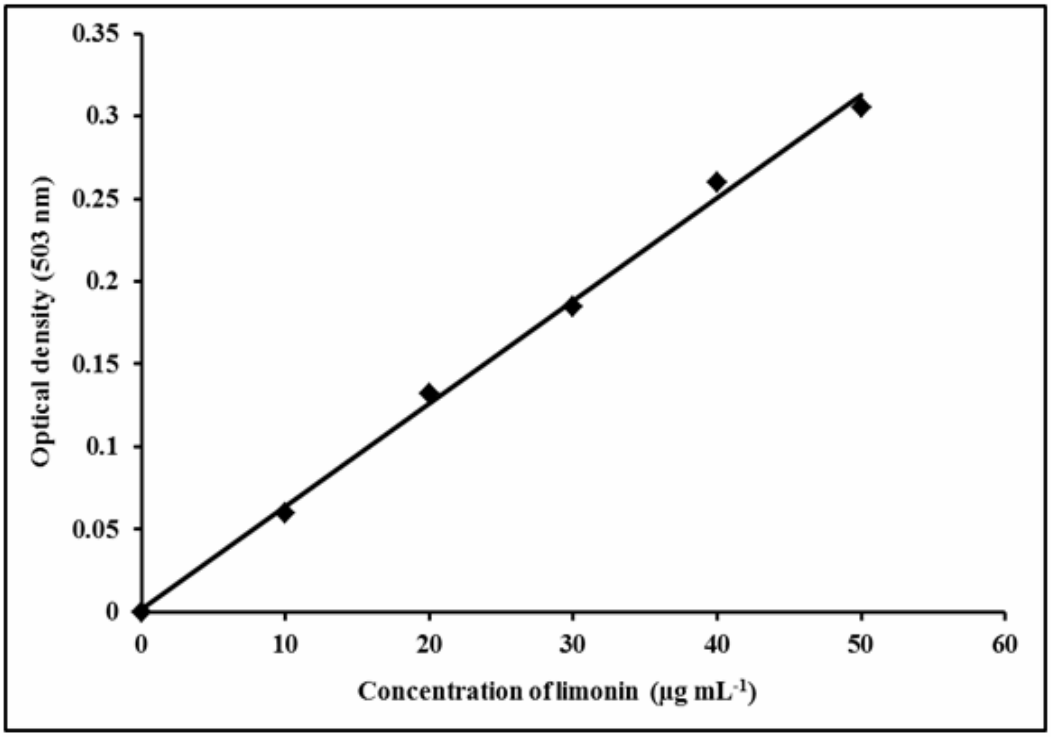


Fig. 3.6 Standard curve of limonin

3.10.9.1.10 Naringin estimation

Naringin was estimated by the the method of Davis (1947) Standard solutions of naringin (1000 ppm stock solution) was prepared by dissolving 100 mg of naringin in 100 mL of warm distilled water. Dilute with distilled water to make 100, 200, 300, 400, 500, and 600 ppm standard solutions **(Fig. 3.7)**. To 0.5 mL of sample or naringin solution 5 mL of 90% DEG (diethylene glycol) was added and mixed well. It was incubated at 27 °C for 10 min, 0.5 mL of 4N NaOH was then added and again mixed well. It was left undisturbed for 15 min. The optical density of resulted yellow color was measured at 420 nm.

3.10.9.1.11 Alcohol estimation

Percent Alcohol (v/v) was calculated by the Spectrophotometric determination method of ethanol (Caputi *et al* 1968) **(Fig. 3.8)**. One mL of fermented wash was taken in 500 mL pyrex distillation flask containing 30 mL of distilled water. The distillate was collected in 50 mL flask containing 25 mL of Potassium dichromate solution (33.768 g of $K_2Cr_2O_7$ dissolved in 400 mL of distilled water with 325 mL of sulphuric acid & volume raised to 1 L). About 20 mL of distillate was collected in each sample and flasks were kept in water bath

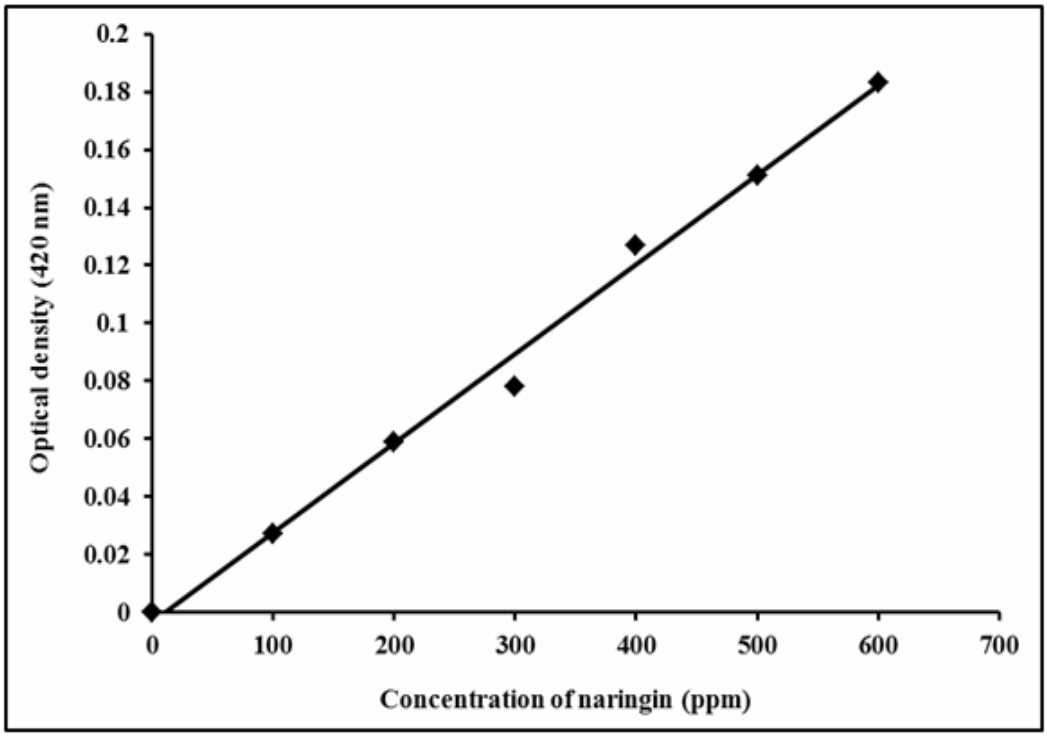


Fig. 3.7 Standard curve of naringin

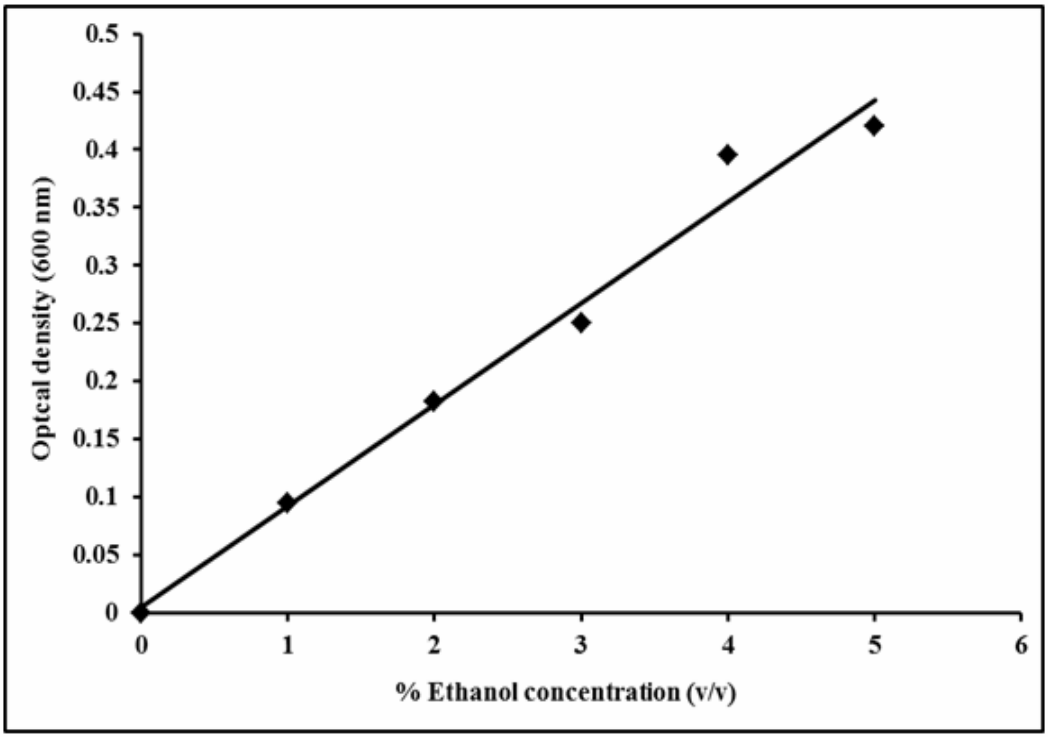


Fig. 3.8 Standard curve of ethanol

maintained at 62.5 °C for 20 min. The flasks were cooled to room temperature and the volume raised to 50 mL. Five mL of this was diluted with 5 mL of distilled water for measuring the optical density at 600 nm using a VIS Double Beam Spectro 1203 (Systronics).

A standard curve was prepared under similar conditions by using standard solution of ethanol containing 1-5% (v/v) ethanol in distilled water.

3.10.9.1.12 Carbon Dioxide volumes determination

Carbon Dioxide volumes in beverage bottles were determined by Zahm and Nagel piercing device. The rationale for this method of carbon dioxide determination was the measurement of the equalized head space pressure at a given temperature.

3.10.9.2 Microbiological analysis

Total yeast count was enumerated on GYE agar by serial plate dilution method.

3.10.9.3 Sensory evaluation

The organoleptic evaluation of kinnow beverages (stored at refrigerated and room temperature) and was done on the basis of appearance, taste, color, aroma, bouquet, body, flavor, astringency and overall acceptability by a panel of judges. Consumer acceptance for the products was evaluated on a nine point "Hedonic scale" (Amerine *et al* 1965) with following scale:

Scale	Sensory Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like/Dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

3.11 Extraction of Pectin

Fresh kinnow peels were collected after juice extraction, dried in hot air oven at 65 °C and grinded to get fine powder. Dried kinnow peel powder (25 g) was blended with 150 mL distilled water. The water to be used for extraction was acidified using 40% citric acid and pH was maintained at 1.2. The acidified mixture of blended peel powder was then heated at 60 ºC for around 120 min. After the heating period was over, the mixture was passed through two fold muslin cloth and was cooled to room temperature. Isolation of pectin was carried out using ethyl alcohol as precipitating agent. Ethyl alcohol was used as a precipitating agent for pectin. Following that, concentrated pectin extracts were precipitated in 95% ethanol. One volume of extracts added in various volume of ethanol. The kinnow peel extracts and ethanol ratios (ER) 1:0.5, 1:1, 1:1.5 and continuous stirring was done for 15 min. Then the mixture was kept aside for 2 h without stirring. Pectin was filtered through four layered muslin cloth. The precipitate was washed 2 to 3 times by ethyl alcohol, to further remove any remaining impurity. Finally, precipitate was kept for drying at 35 ºC to 40 ºC in hot air oven. It was then stored in desiccators until further use (Menon *et al* 2011).

3.12 Statistical analysis

Statistical analysis was done by using CPCS1 software. Experimental data were analyzed using standard analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Standard errors were calculated for all mean values. Differences were considered significant at the $p \leq 0.05$ level. Binary data were used to calculate Jaccard similarity coefficient and cluster analysis was performed using NTSYSpc version 2.0 (Saitou and Nei 1987). Evolutionary distance was calculated using the method of Jukes and Cantor (1969).

Chapter - IV

RESULTS AND DISCUSSION

4.1 Isolation and screening of yeast isolates for α-L-rhamnosidase production

A total of thirty, morphologically different yeast colonies were isolated from the whey beverage during streak purification and discovered diverse colony type. The production of rhamnosidase enzyme activities were screened for all isolates by the liquid culture (GYE-broth, pH 6) inoculated with a loopful of 24 h old actively growing culture and incubated at 28±2 °C in an orbital shaker at 200 rev min^{-1} for 24-36 h with 0.2% inducer naringin. Out of thirty isolates, only four isolates (code- 84, B-82, 86, S-82) showed α-L-rhamnosidase enzyme production. Results were recorded according to the IU mL^{-1} enzyme produced by assay production. Isolate 84 was able to produce maximum α-L-rhamnosidase followed by B-82, 86 and S-82 respectively. All four isolated produced α-L-rhamnosidase in the range of 0.029±0.00 to 0.058±0.02 IU mL^{-1}. Isolate 84 produced the maximum activity at 0.058±0.02 IU mL^{-1} and S-82 produced minimum activity (0.029±0.00 IU mL^{-1}) (**Plate 4.1, Table 4.1**).

Kaminarides and Laskos (1992) isolated 128 yeast colonies from whey beverage; i.e., *Saccharomyces* (59%), *Candida* (17%) and *Pichia* (12%) and suggested that yeasts play an important role in the preservation of feta cheese and attributes to chemical and organoleptic qualities. A pure colony of yeast isolate, *Candida colliculosa* was also isolated from whey beverage (Jooyandeh 2013). The 386 yeast strains had been examined for their α-L-rhamnosidase activity using L-rhamnose as the inducer (Yanai and Sato 2000). The screening results showed that low levels of α-L-rhamnosidase activities were observed in *Saccharomyces cerevisiae*, *Cryptococcus terreus*, *Pichia angusta* and *Pichia capsulate* (IU mL^{-1}- 0.0137, 0.0065, 0.034 and 0.0288) as compared to present study. Some yeast like *Hansenula, Debaryomyces, Candida and Aureobasidium pullulans* also showed low levels of α-L-rhamnosidase activities (McMahon *et al* 1999). The main sources of this enzyme are filamentous fungi (Monti *et al* 2004), have shown the induction of α-L-rhamnosidases production in the number of fungal strains such as *Acremonium persicinum*, *Circinella muscae*, *Emericella nidulans*, *Fusarium oxysporum*, *Mortierella alpina*, *Penicillium oxalicum*, *Rhizopus arrhizus*, *Talaromyces flavus* and *Trichoderma harzianum*, using L-rhamnose, naringin, rutin, hesperidin as inducers. A number of *Aspergillus* species (*Aspergillus niger, A. terreus, A. nidulans* and *A. aculeatus*) have been reported for the production of α-L-rhamnosidases. Some bacterial strains producing α-L-rhamnosidases are thermophilic bacteria (Birgisson *et al* 2004), *Fusabacterium* (Park *et al* 2005), *Pseudoalteromonas species, Ralstonia pickettii* (Orrillo *et al* 2007), *Lactobacillus acidophilus*

(Beekwilder *et al* 2009), *Pediococcus acidilactici* (Michlmayr *et al* 2011), *Clostridium stercorarium*, *Sphingomonas paucimobilis*, *Bacillus sp.* and *Corticium rolfsii.*

Table 4.1: Rhamnosidase activity assay of different yeast isolates

Sr. No.	Culture Code	Enzyme Activity (IU mL^{-1})
1	84	0.058±0.02
2	B-82	0.046±0.01
3	86	0.033±0.01
4	S-82	0.029±0.00

Mean values±standard error of three independent experiments

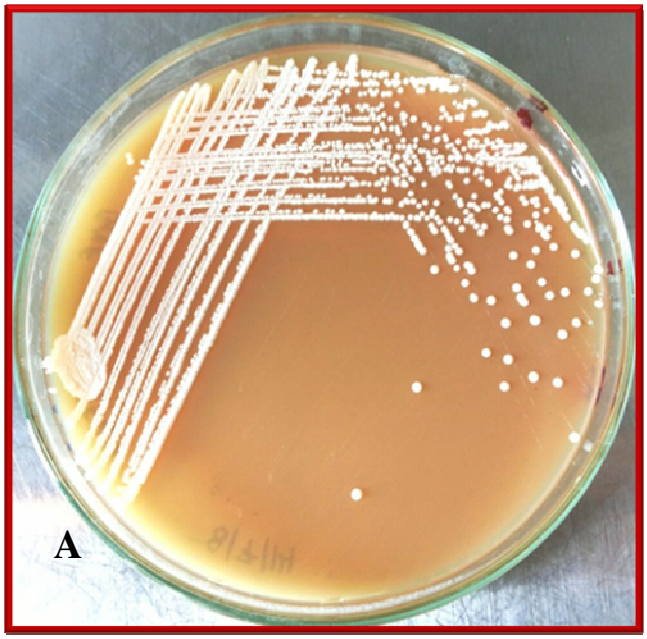
4.2 Morphological and biochemical characterization of the α-L-rhamnosidase producing yeast isolates

Phenotypic characterization of all four yeast isolates showed that most of them were cream coloured, smooth colony and circular in shape. All isolates were able to grow at pH range between of 3 to 6.5 and temperature between 20 to 45 °C while one isolate (84) could tolerate maximum pH range (3-6.5) and temperature tolerance (20-45 °C). Isolate 84 showed the maximum range of salt tolerance of 2 to 6% of NaCl concentration. It required for the minimum period of incubation time i.e., 20-24 h **(Table 4.2)**. Morphological results confirmed the close relationship of the yeast isolates.

Isolates 84 was found to be resistant to 0.1% cycloheximide concentration (0.1% v/v) and could endure high osmotic pressure (60% glucose). Isolates, B82, S82 and 86 were able to resist cycloheximide concentration of 0.01% (v/v) only. Biochemical tests results indicated that all isolates exhibited negative test for lipase, urea hydrolysis, acid production from glucose, starch and were not able to grow in vitamin free medium **(Table 4.2)**.

The α-L-rhamnosidase producing isolates (84, B82, 86 and S82) were able to ferment carbon sources such as D-glucose, D-galactose, D-raffinose, D-xylose and sucrose but could ferment nitrogen sources as ethylamine and L-lysine whereas nitrate, nitrite and D-glucosamine were not assimilated **(Table 4.2)**.

In a similar study conducted by Karasu-Yalcin *et al* (2012), the authors reported that all 17 different yeast isolates showed no growth at 60% glucose, on vitamin free media and showed negative urea hydrolysis test. Only two strains showed growth at 50% glucose, three at 37 °C temperature and five were able to ferment glucose. Ghosh (2011) studied the growth of 13 different yeast isolates at different temperatures (25, 30, 35, 37 and 40 °C, respectively) and reported that all strains showed growth up to 35 °C, nine up to 37 °C and four up to 40 °C. Out of 13, three exhibited positive urea hydrolysis test, four fermented glucose and showed growth on at 0.01 and 0.1 % cycloheximide concentration.



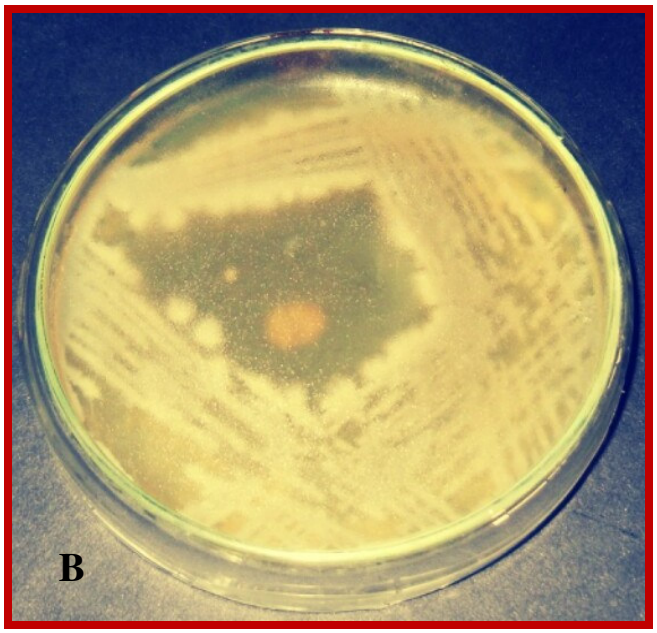


Plate 4.1 Morphology of yeast strain 84 on GYE media
(A) Absence of naringin (B) Presence of 0.2% naringin

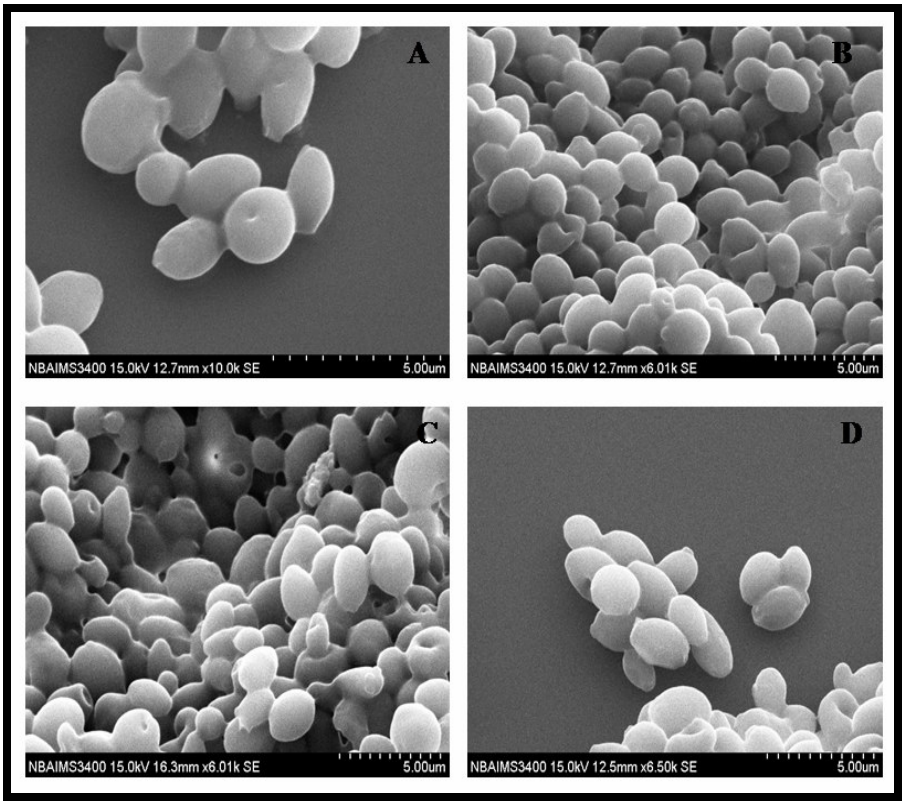


Plate 4.2 Scanning electron micrographs of yeast strains producing rhamnosidase enzyme (A) 84 (B) B-82 (C) 86 (D) S-82

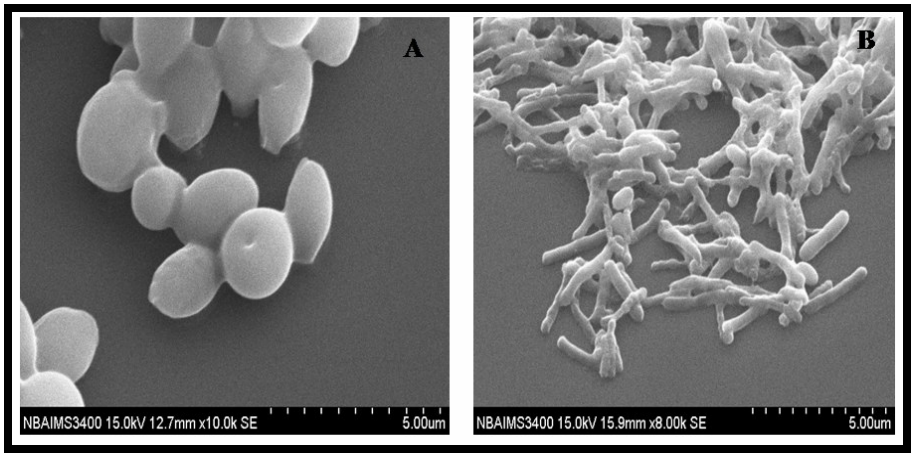


Plate 4.3 Scanning electron micrographs of yeast strain 84 producing rhamnosidase enzymes (A) Absence of naringin (B) Presence of 0.2% naringin

Table 4.2: Characterization of α-L-rhamnosidase producing yeast isolates

	Yeast isolates			
Characteristics	**84**	**B-82**	**86**	**S-82**
Morphological parameters				
Colony colour	Cream	Cream	Cream	Cream
Colony	Smooth	Smooth	Smooth	Smooth
Shape	Circular	Circular	Circular	Circular
Filamentous	ND	ND	ND	ND
Pigmentation	ND	ND	ND	ND
Ascospore	Present	Present	Present	Present
Growth parameters				
Temperature (°C)	20-45	20-40	20-40	20-40
pH	3-6.5	3-6	3-6	3-6
Salt (%)	2-6	2-5	2-5	2-5
Glucose (%)	60	50	50	50
Time (h)	20-24	20-24	24-36	24-36
Biochemical parameters				
Lipase	-	-	-	-
Urea hydrolysis	-	-	-	-
Acid production from glucose	-	-	-	-
Starch	-	-	-	-
Cycloheximide (%)	0.01 and 0.1	0.01	0.01	0.01
Vitamin free medium	-	-	-	-
Fermentation parameters				
Carbon sources:				
1. D- glucose	+	+	+	+
2. D-galactose	+	+	+	+
3. D- raffinose	+	+	+	-
4. D- xylose	+	+	+	-
5. Sucrose	+	+	+	+
Nitrogen sources:				
1. Ethylamine	+	+	+	+
2. L- lysine	+	+	+	+
3. Nitrate	-	-	-	-
4. Nitrite	-	-	-	-
5. D- glucosamine	-	-	-	-

* ND- Not determined

4.3 Visible effect of yeast isolates through scanning electron microscopy

Scanning electron microscopy images depicts the cell morphology of α-L-rhamnosidase producing isolates during growth in Glucose Yeast Extract medium after 4 days at 28±2 °C **Plate 4.2**. Isolate 84 was further cultured with and without 0.2% inducer naringin and different morphotypes of yeast by inducer were observed **(Plate 4.3)**. Similar studies were performed by Kurtzman and Fell (1997) by scanning electron microscopy, studying morphology of different yeast strains grown in YPD medium. They observed that *Candida*

spp. cells are globose, ellipsoidal, cylindroidal or elongate, occasionally ogival, triangular of lunate. *Kluyveromyces* spp. cells are ovoidal, ellipsoidal, cylindrical to elongate and *Saccharomyces* spp. cells are globose, ellipsoidal or cylindroidal.

4.4 Metabolic characterization of the yeasts

On the basis of the database from Biology system, the sole carbon sources utilization pattern of rhamnosidase producing isolates were formed **(Fig. 4.1)**. The results indicate that isolates 84, B82, 86 and S82 assimilated and utilized 44, 42, 39 and 37 types of different carbon sources respectively. Among the substrate utilization, the order is carbohydrate followed by the carboxylic acids, amino acids, polymers and other miscellaneous carbon classes **(Table 4.3, Plate 4.4)**. Based on the fermentation and carbon source assimilation spectra of the marine yeast W14-3, Wang *et al* (2008) could report that the yeast strain was closely related to *C. membranifaciens*. Biolog analysis shows that it could assimilate glucose, galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, melibiose, raffinose, inulin, D-xylose, L-arabinose, D-arabinose and L-rhamnose but could not assimilate L-rhamnose. Praphailong *et al* (1997) also used Biolog system for the identification of 21 species (72 strains) of yeasts of food and wine origin.

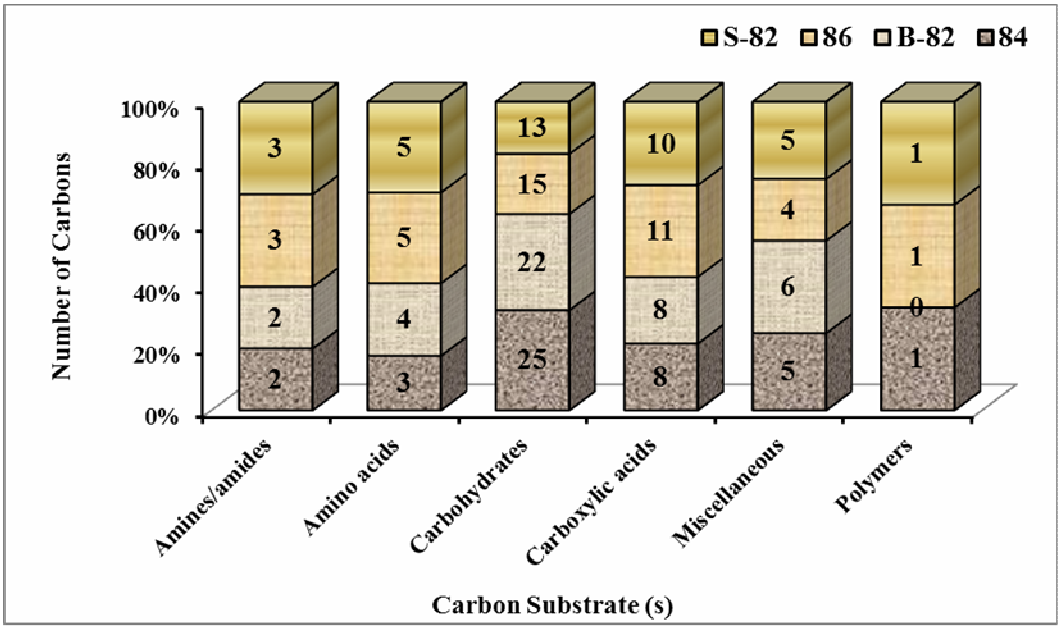


Fig. 4.1 Metabolic characterization of the α-L-rhamnosidase producing yeast strains using Biolog

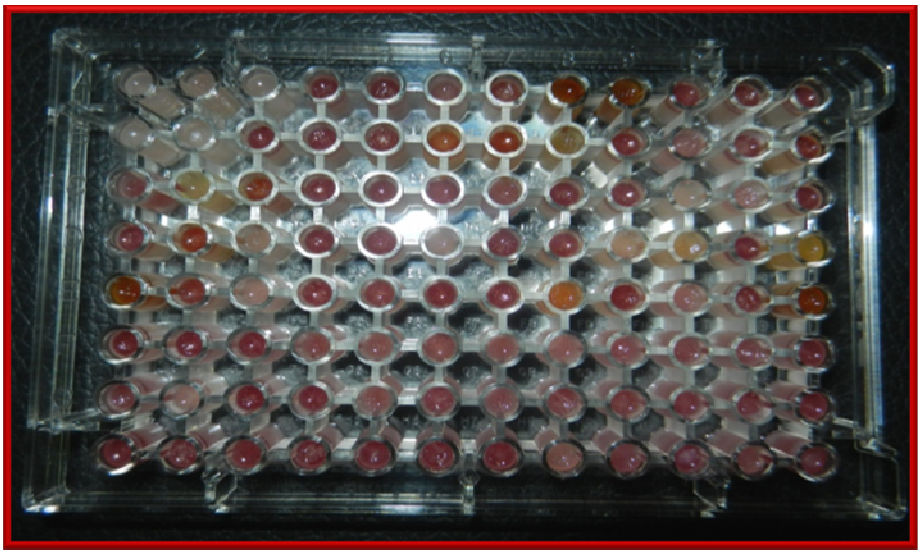


Plate 4.4 Biolog plate

Table 4.3: Carbon source utilization profile of α-L-rhamnosidase producing yeast

	Substrate	84	B-82	86	S-82
Amines/amides	2-Amino Ethanol	_	_	+	+
	D- Glucosamine	_	_	_	_
	Glucuronamide	+	+	+	+
	L-Alaninamide	_	_	_	_
	Putrescine	_	_	+	+
	Succinamic Acid	+	+	_	_
Amino acids	y-Amino-butyric-acid	_	_	_	_
	Glycyl-L-Glutamic Acid	_	_	_	_
	L-Alanine	+	+	_	_
	L-Alanyl-Glycine	_	_	_	_
	L-Asparagine	_	_	_	_
	L-Aspartic Acid	_	_	_	_
	L-Glutamic Acid	_	_	_	_
	L-Ornithine	_	_	_	_
	L-Phenylalanine	_	_	+	+
	L-Proline	+	+	+	+
	L-Pyroglutamic Acid	+	+	+	+
	L-Serine	_	+	+	+
	L-Threonine	_	_	+	+
Carbohydrates	α- D- Glucose	+	+	+	+
	α- D- Lactose	_	_	_	_
	α- Methyl- D- Galactoside	+	+	_	_
	β - Methyl- D- Galactoside	_	_	_	_
	α- Methyl- D- Glucoside	_	_	_	_
	β - Methyl- D- Glucoside	_	_	_	_
	Adonitol	_	_	_	_
	Arbutin	+	+	+	_
	D- Arabinose	+	+	+	+
	D- Arabitol	+	+	_	_
	D- Cellobiose	_	_	+	_
	D- Fructose	+	+	+	+
	D- Galactose	+	+	+	+
	D- Mannitol	+	+	_	_
	D- Mannose	+	+	+	+

	D- Melezitose	+	+	_	_
	D- Malibiose	+	+	_	_
	D- Psicose	_	_	_	_
	D- Raffinose	_	_	_	_
	D- Ribose	+	+	+	+
	D- Sorbitol	_	_	_	_
	D-Tagatose	_	_	+	+
	D-Trehalose	_	_	_	_
	D-Xylose	_	_	+	+
	Gentiobiose	+	+	_	_
	i- Erythritol	+	_	_	_
	Lactulose	+	+	_	_
	L- Arabinose	+	+	+	+
	L- Fructose	+	+	+	+
	L- Rhamnose	+	+	+	+
	L- Sorbose	_	_	+	+
	Maltitol	+	+	_	_
	Maltose	+	+	_	_
	Maltotriose	+	_	_	_
	m- inositol	+	+	_	_
	N-acetyl-D- Galactosamine	_	_	_	_
	N- acetyl-D- Glucosamine	+	+	+	+
	N- acetyl-D-Mannosamine	+	_	_	_
	Palatinose	_	_	_	_
	Sedoheptulosan	_	_	_	_
	Stachyose	_	_	_	_
	Sucrose	_	_	_	_
	Turanose	_	_	_	_
	Xylitole	+	+	_	_
Carboxylic acids	β -Hydroxy-butyricAcid	_	_	+	+
	y-Hydroxy-butyricAcid	_	_	+	+
	α -Keto-glutaricAcid	+	+	+	+
	2- Keto- D- Gluconic acid	_	_	+	+
	D- Galacturonic Acid	+	+	+	+
	D- Gluconic Acid	+	+	+	+
	D- Glucuronic Acid	+	+	+	+

	D-Malic Acid	+	+	+	+
	D-Saccharic Acid	_	_	+	+
	Fumaric acid	_	_	_	_
	L-Lactic Acid	_	_	_	_
	L-Malic Acid	_	_	+	+
	N-Acetly-L-Glutamic Acid	+	+	_	_
	p-Hydroxyphenylaceticacid	+	+	_	_
	Quinic Acid	+	+	+	+
	Sebacic Acid	_	_	_	_
	Succinic Acid	_	_	_	_
Miscellaneous	Adenosine	_	_	_	_
	Amygdalin	_	_	_	_
	Adenosine-5'- Monophosphate	_	+	+	+
	Bromosuccinic acid	+	+	_	_
	D-LacticAcidMethyl Ester	_	_	+	+
	Glucose-1- Phosphate	+	+	+	+
	Glycerol	+	+	_	_
	Salicin	_	_	_	_
	Succinic Acid Mono-Methyl Ester	+	+	+	+
	Uridine	+	+	_	+
Polymers	α- Cyclodextrin	_	_	+	_
	β- Cyclodextrin	_	_	_	_
	Dextrin	+	_	_	_
	Glycogen	_	_	_	_
	Tween 80	_	_	_	+

4.5 Molecular characterization, accession numbers and phylogenic tree analysis of yeasts

Sequence analysis of phylogeny for microbial taxonomy is a more accurate method for determining inter- and intra-specific relationships because traditional identification methods, depending on phenotypes, usually lead to uncertain and inaccurate interpretations of species interaction (Kurtzman and Fell 2000). So, based on 18S rRNA gene partial sequencing, similarity values of ≥97% suggested that isolates were characterized as *Clavispora lusitaniae* (84), *Clavispora lusitaniae* (B82), *Candida* sp. YS12A (86) and *Candida hyderabadensis* (S82). Partial 18S rRNA gene sequences of the strains had been submitted to NCBI GeneBank under accessions numbers KF633446, KF633447, KF680225 and KF680226 **(Plate 4.5, Table 4.4, Fig. 4.2)**.

Table 4.4: Molecular identification of α-L- rhamnosidase producing yeast strains isolated from whey beverage based on 18S rRNA gene sequence

Isolate code	Source	Yeast strains identified	Similarity (%)	NCBI accession Number	Amplicon size (bp)	BOX Bands	ERIC bands	REP bands
84	Whey beverage	*Clavispora lusitaniae*	EF221824 (100 %)	KF633446	334	14	7	8
B82	Whey beverage	*Clavispora lusitaniae*	EF568047 (100 %)	KF633447	337	11	7	5
86	Whey beverage	*Candida* sp. YS12A	DQ857760 (100 %)	KF680225	524	8	5	4
S82	Whey beverage	*Candida hyderabadensis*	AM180949 (100 %)	KF680226	309	10	7	6

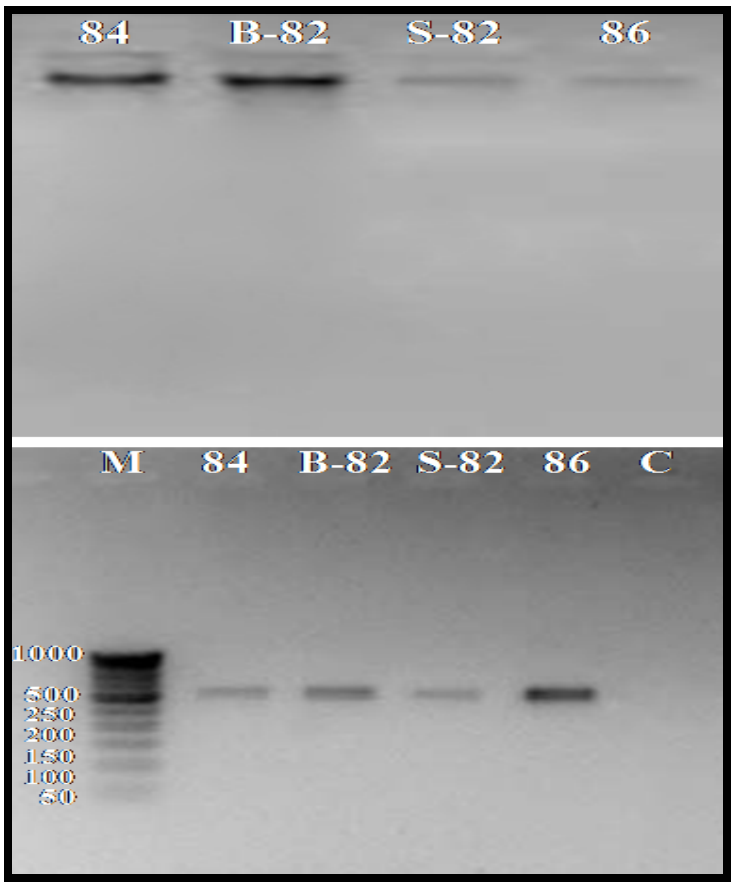


Plate 4.5 (A) Genomic DNA isolated from the α-L-rhamnosidase producing yeast strains

(B) Amplification of 18S rRNA gene from the α-L-rhamnosidase producing yeast strains using universal primers (M, molecular size marker (50 bp-1000 bp) and low range DNA ruler (GeNeiTM)

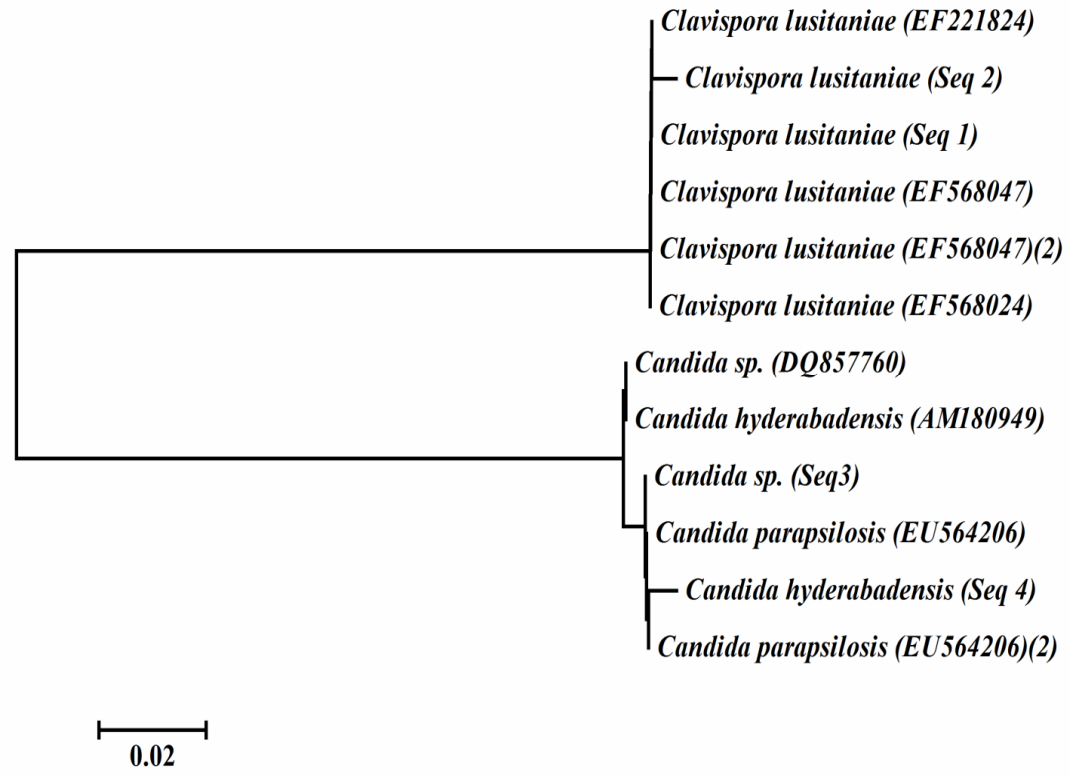


Fig. 4.2 Phylogenetic tree of partial ITS rRNA gene sequences of rhamnosidase producing isolates with those of maximum similar entries from NCBI database. The tree is created by neighbor-joining method with 1,000 bootstrap re-samplings; values lower than 50 are not shown. Scale bar represents the number of changes per base position

Seq 1 *Clavispora lusitaniae* strain XJURML-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence

>TTAAAAAATACATTACACATTGTTTTTGCGAACAAAAAAATAAATTTTTTTATTC
GAATTTCTTAATATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGA
AGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCTCGAGGCATTCCTCGAGGCATGCCTGTTTGAGCGT
CGCATCCCCTCTAACCCCCGGTTAGGCGTTGCTCCGAAATATCAACCGCGCTGTC
AAACACGTTTACAGCACGACATTTCGCCCTCAAATCAGGTAGGACTACCCGCTGA
ACTTAAG

Seq 2 *Clavispora lusitaniae* strain WM 02.466 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

>TTAAAAAATACATTACACATTGTTTTTGCGAACAAAAAAATAAATTTTTTTATTC
GAATTTCTTAATATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGA
AGAACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGGAATCATCGAATC
TTTGAACACATTGCGCCTCCGAGGCATTCCTCGAGGCATGCCTGTTGAGCGTCGC
ATCCCCTCTAACCCCCGGTTAGGCATTGCTCCGAAATATCAACCGCGCTGGTCAA
ACACGTTTACAGCCAGCACGACATTTCGCCCTCAGGTAGGACTACCCGCTGAACT
TAAG

Seq 3 *Candida sp.* YS12A 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

>TTTCAGTAGGTGACCTGCGGAGGATCATTACAGAATGAAAAGTGCTTAACTGCA
TTTTTTCTTACACATGTGTTTTTCTTTTTTTGAAAACTTTGCTTTGGTAGGCCTTCT
ATATGGGGCCTGCCAGAGATTAAACTCAACCAAATTTTATTTAATGTCAACCGAT
TATTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
AACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGC
GTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTT
GAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTGGTACAA
ACTCCAAAACTTCTTCCAAATTCGACCTCAAATCAGGTAGGACTACCCGCTGAAC
TTAAGCATATCAATAAGCGGAGGAAAAG

Seq 4 *Candida hyderabadensis* strain NRRL Y-27953T18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

ATCGATGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATATTCGTGAA

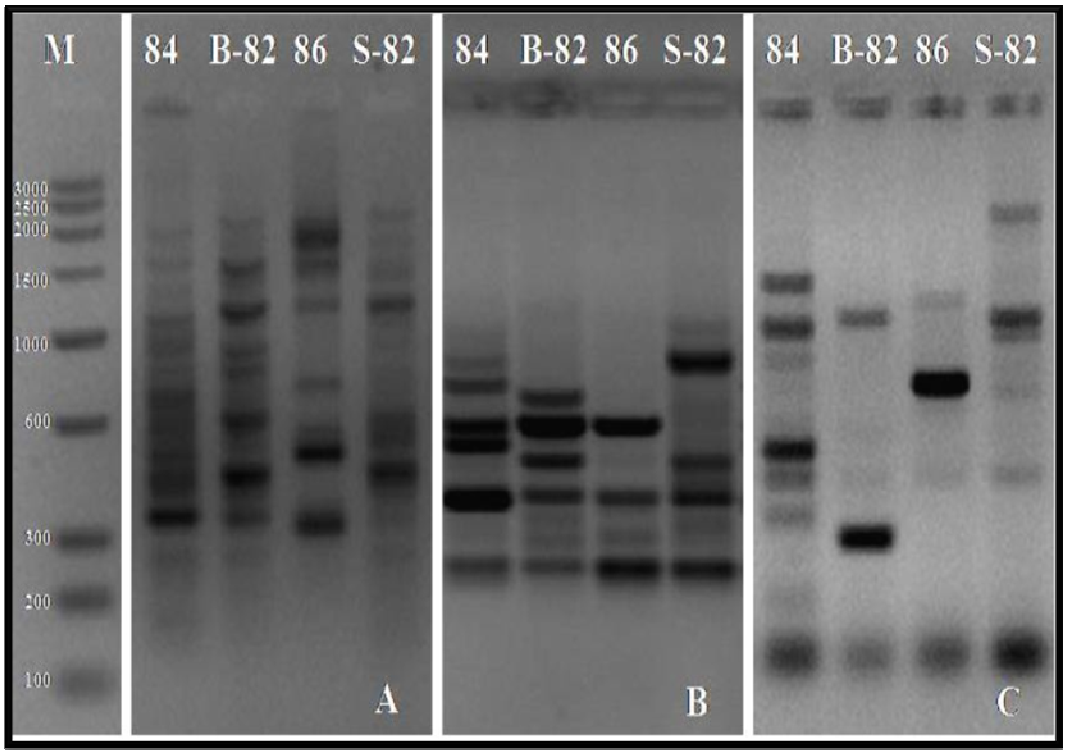


Plate 4.6 Genomic DNA fingerprinting generated from genomic DNA of yeasts. M, molecular size marker (100 bp-3 kb) and low range DNA ruler (GeNeiTM) (A) BOX-PCR (B) ERIC-PCR (C) REP-PCR

TCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGT
TTGAGCCTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGT
TTGCTTGAAAGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTCCACTCATTG
GTACAAACTCCAAAACTTCTTCCAAATTCGACCTCAAATCAGTAGGACTACCCGC
TGAACTTAAGCATATCAATAAGCGGAGGAAAAG

4.6 Genomic DNA fingerprinting using BOX, ERIC and REP-PCR

Distinct banding patterns to distinguish isolate *Clavispora lusitaniae* (84)*, Clavispora lusitaniae* (B82)*, Candida* sp. YS12A (86) and *Candida hyderabadensis* (S82) were generated with each fingerprinting technique using the respective oligonucleotide primers. The amplified bands in BOX consistently revealing more intense and more polymorphic bands compared to ERIC and REP. BOX, ranging from 200 to 2,000 bp. ERIC ranged in length from 250 to 1,500 bp whereas for the REP they ranged from 150 to 2,000. The number and intensity of amplified bands varied with each fingerprint technique, with maximum numbers of amplified bands were shown by *Clavispora lusitaniae* (84) (14, 7, 8) followed by *Clavispora lusitaniae* (B-82) (11, 7, 5), *Candida hyderabadensis* (S82) (10, 7, 6) and *Candida* sp. YS12A (86) (8, 5, 4) in BOX, ERIC and REP-PCR fingerprinting techniques, respectively **(Table 4.4 and Plate 4.6)**. Hierro *et al* (2004) proved that REP and ERIC PCR methods were useful in characterizing 15 different yeast species and strains in wine. They found that REP-PCR method was inadequate for intra-specific characterization of yeast isolates but useful for identification of yeasts. These techniques are an inexpensive way for winemakers and researchers to identify oenological relevant yeast species and can be used to differentiate between *S. cerevisiae* and *S. bayanus* (Hierro *et al* 2004). For the selection of yeast isolates, molecular biology-based grouping of the 125 isolates was performed using repetitive extragenic palindromic (rep- PCR) and cluster was done only by the different profiles obtained from rep-PCR technique (Miguel and Cardoso 2013).

4.7 Growth kinetics studies of yeast *Clavispora lusitaniae* KF633446

4.7.1 Growth kinetics studies of yeast *Clavispora lusitaniae* KF633446 in Glucose Yeast Extract (GYE) broth without naringin

The growth curve of yeast *Cavispora lusitaniae* 84 **(Fig. 4.3)** in GYE broth under aerobic conditions with respect to cell biomas and optical density was found to show normal patterns with first a very short lag period of (1-2 h), the cells increase in size and weight followed by phase of exponential growth up to 48 h as indicated by sharp increase in optical density and dry weight from (0.37- 1.81) and (0.03-0.83) g respectively. This is because of adaptation of yeast in the medium followed by exponential phase, while in stationary phase there was no further shift in either optical density and cell dry weight. After an initial lag phase, exponential growth proceeds primarily by fermentation, whereas respiration is substantially repressed. After an exhaustion of the initial fermentable carbon source, cells

metabolically adapt to respiration using ethanol during second (diauxic) lag phase that is followed by second exponential growth phase, although at a slower rate. When cell start growing on non-fermentable substrate (glycerol), the second lag and exponential phase do not appear and the initial exponential growth is immediately followed by stationary phase. Cell enters the stationary phase as a result of carbon starvation, but exhausting other nutrients, including nitrogen, phosphorus and sulfur also force cells to enter stationary phase. Stationary phase cells are characterized by altered physiological properties, exhibit increased resistance to various stresses.

The growth curve for cell biomass shows a normal pattern with first a slow increase in cell dry weight (0.03-0.9 g/100 mL). The decline phase is the result of the inability of the yeast to carry out further reproduction as condition in the medium become less supportive for cell division. Industrial fermentation is usually interrupted at the end of the exponential growth phase or before the death phase begins.

4.7.2 Growth of yeast *Clavispora lusitaniae* 84 in Glucose Yeast Extract broth (GYE) with naringin

The overall growth kinetics of *Clavispora lusitaniae* (84) with and without inducer naringin demonstrating similar lag, log-growth and stationary phase. But the growth rate of *Clavispora lusitaniae* grown in GYE medium with inducer naringin (0.2%) is fast as compared to growth rate of yeast in medium without inducer **(Fig. 4.5)**. When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present. The rate of growth also increases with nutrient concentration but in a hyperbolic manner much like that seen with many enzymes. The shape of the curve seems to reflect the rate of nutrient uptake by microbial transport proteins. The growth curve of yeast in GYE broth with inducer naringin with respect to cell biomass had been found to show normal patterns with first a very short lag period followed by phase of exponential growth upto 40 h. The growth curve for cell biomass also show a normal pattern with first a slow increase in dry weight from (0.05-0.13) g/100 mL up to 24 h and then a sharp increase from 24-48 h (0.13- 0.79) g/100 mL, followed by stationary phase in which biomass did not increased significantly after 48-72 h (0.81-0.98) g/100 mL. Death of cells occurs under natural conditions and can be boosted by adjusting the environmental factors beyond the degree of tolerance of microbial cells **(Fig. 4.4)**.

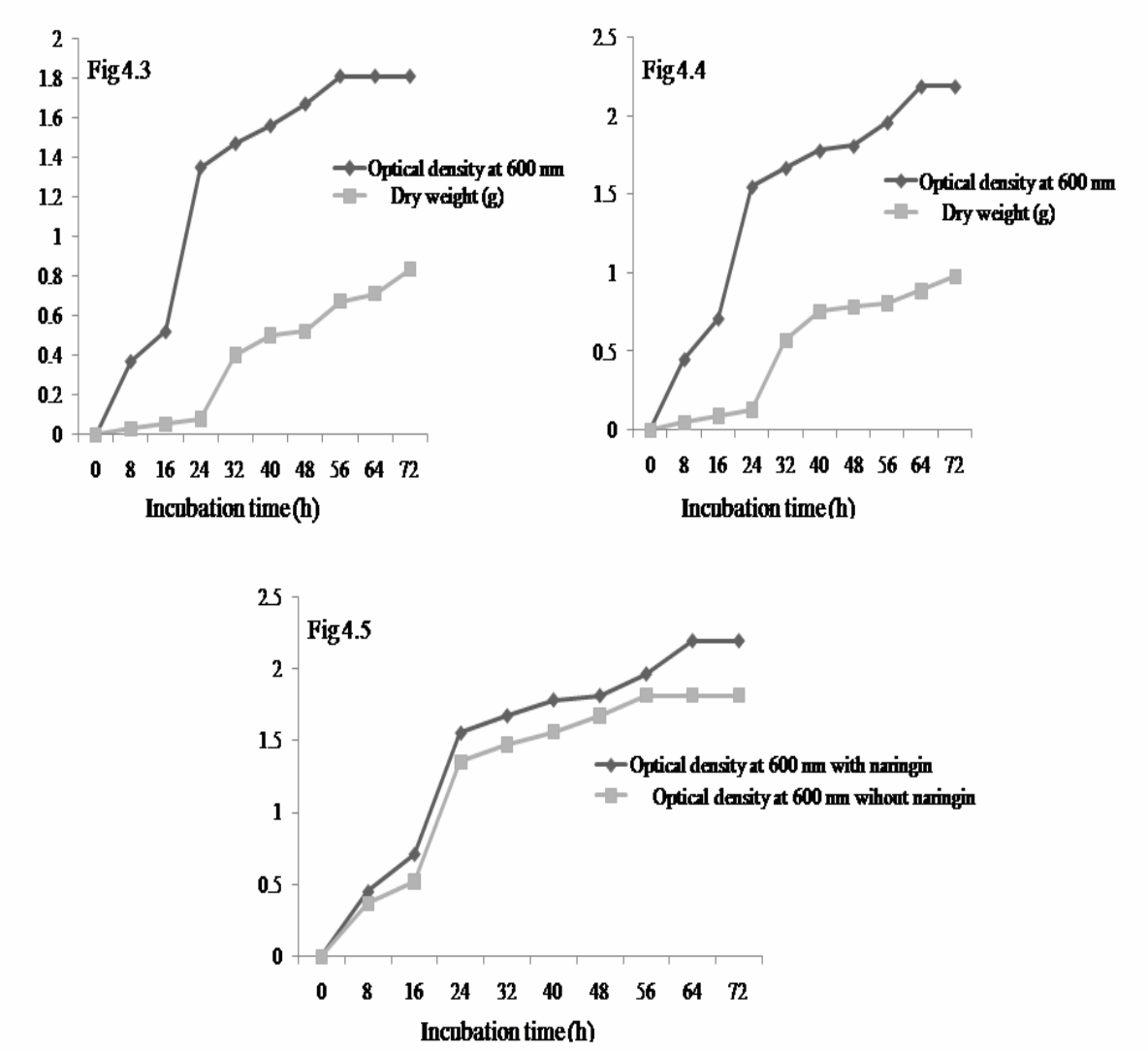


Fig. 4.3 Growth of yeast *Clavispora lusitaniae* KF633446 in GYE broth without naringin

Fig. 4.4 Growth of yeast *Clavispora lusitaniae* in GYE broth with inducer naringin

Fig. 4.5 Comparison of growth of yeast *Clavispora lusitaniae* KF633446 with and without inducer naringin

4.8.1 Screening of media components for optimization of α-L-rhamnosidase production

4.8.1.1 Effect of carbon source on α-L-rhamnosidase production

A differential response in rhamnosidase activity had been obtained due to supplementation of various carbon sources. Among various carbon sources, rhamnose exhibited maximum enzyme activity (0.056 IU mL^{-1}) and glucose exhibited minimum rhamnosidase activity (0.016 IU mL^{-1}) after 48 h of incubation **(Fig. 4.6)**. Further, optimization of rhamnose concentration (0.1-1%-w/v), it had been found that *Clavispora lusitaniae* KF633446 produced maximum enzyme (0.065 IU mL^{-1}) when grown on medium containing 0.6% rhamnose as compared to other concentrations **(Fig. 4.10, Table 4.5)**. Yeast

strains *Saccharomyces cerevisiae*, *Cryptococcus terreus*, *Pichia angusta* and *Pichia capsulate* showed low levels of α-L- rhamnosidase activity (IU mL^{-1}- 0.0137, 0.0065, 0.034 and 0.0288) in presence of rhamnose as compared to present yeast strain (Yanai and Sato 2000). Similar results was observed by Elinbaum *et al* (2002) that rhamnose could be used as an inducer in the production of *Aspergillus terreus* α-L-rhamnosidase by solid state fermentation, however they reported that naringin was a better inducer than rhamnose. Puri *et al* (2005) reported that naringinase activity was repressed by glucose, sucrose and lactose although these carbon sources supported excellent growth. Production of α-L-rhamnosidase by *A. kawachii* is mediated by carbon catabolite repression (Koseki *et al* 2008). They found that α-L-rhamnosidase production by *A. kawachii* was significantly induced in presence of 0.5% L-rhamnose, but the production was repressed in presence of 0.5% L-rhamnose supplemented with 1% glucose and enzyme was not produced when *A. kawachii* was grown on 0.5% glucose as the sole carbon source. Puri *et al* (2005) observed rhamnose and molasses (10 g L^{-1}) exhibited highest naringinase activity (4.6 IU mL^{-1}) in salt medium with naringenin after 8 days of fermentation (Puri *et al* 2005). In present study yeast strain *Clavispora lusitaniae* KF633446 produces α-L-rhamnosidase in short duration fermentation (2 days) as compared to reported fungal strains (7-10 days). The reduction in fermentation time is important because it decreases the fermentation costs and contamination with opportunistic microorganisms in scale up process.

4.8.1.2 Effect of nitrogen source on α-L-rhamnosidase production

The effect of different nitrogen sources was tested for rhamnosidase production in minimal medium containing 0.2% naringin supplemented with 0.6% (w/v) rhamnose. Results indicated that minimal medium with yeast extract showed maximum rhamnosidase activity (0.057 IU mL^{-1}) followed by peptone (0.050 IU mL^{-1}), casein (0.047 IU mL^{-1}), urea (0.038 IU mL^{-1}), ammonium sulphate (0.035 IU mL^{-1}) and ammonium chloride (0.024 IU mL^{-1}) as a nitrogen source during 48 h of incubation **(Fig. 4.7)**. Further, varying concentration of yeast extract (0.1-1%-w/v) tested and revealed, 0.4% (w/v) yeast extract producing highest rhamnosidase activity **(Fig 4.11, Table 4.6)**. In similar study, yeast extract (Bram and Solomons 1965) and peptone (Chen *et al* 2010; Puri *et al* 2005) were able to increased the production of naringinase enzyme. Peptone was the most effective in naringinase biosynthesis from *Aspergillus niger* (Puri *et al* 2005) and *Aspergillus oryzae* JMU316 (Chen *et al* 2010). In terms of the enzyme yield, the optimum concentration of peptone was 5 g L^{-1} and higher concentrations of peptone in the fermentation medium did not significantly increase enzyme yield (Puri *et al* 2005). Inorganic nitrogen sources yielded low naringinase production in shaking-flask cultures relative to organic sources (Norouzian *et al* 2000). Inorganic nitrogen sources could only marginally synthesize certain essential amino acids in fermentation by fungi and organic nitrogen sources were favorable for metabolite production (Hwang *et al*

2003; Kim *et al* 2003). The maximum naringinase production of *Aspergillus niger* BCC 25166 obtained by supplementing medium with $NaNO_3$ as its nitrogen source (Thammawat *et al* 2008). Urea and diammonium hydrogen phosphate were inhibitory, presumably because of the release of ammonium ions (Puri *et al* 2005).

4.8.1.3 Effect of temperature on rhamnosidase activity

In case of temperature optimization, maximum α-L- rhamnosidase activity (0.05 IU mL^{-1}) was observed at temperature 35 °C after 48 h of incubation and decreased slowly and decreased slowly with increase in temperature upto 50 °C **(Fig. 4.8)**. The reason for the decrease in enzyme activity above and below the 35 °C temperature may due to deactivation of enzyme by weakening of non covalent interactions that stabilize the protein structure, leading to unfolding and subsequent changes and reduction in catalytic activity of enzyme. This suggests that the temperature for enzymatic hydrolysis of naringin and conversion of other flavonoids should be controlled at 35 °C. Optimum temperature for α-L- rhamnosidase activity of *Pichia angusta* (Yanai and Sato 2000) and *Aspergillus nidulans* (Orejas *et al* 1999) was observed at 40 °C. Yadav and Yadav (2004) found that optimum temperature of α-L-rhamnosidases from the different *Aspergillus* strains vary from 53-60 °C. The temperature optimum for naringinase activity was 50 °C for *Bacillus methylotrophicus* (Mukund *et al* 2014) and *Aspergillus niger* MTCC1344 (Thammawat *et al* 2008).

4.8.1.4 Effect of pH on rhamnosidase activity

The effect of pH on yeast α-L- rhamnosidase activity was tested in a range of 3 to 8 and pH 4 showed maximum activity (0.05 IU mL^{-1}) as compared to pH 5, 6, 7, 8 and 3 (**Fig. 4.9**). The reason for decrease in enzyme activity above and below pH 4 may be the change in enzymatic structure due to altering of amino acids. The increase in production at low pH level is of great importance in fruit juice processing industry because pH of juice is less than 5. Additionally, low pH reduces the chances of bacterial contamination in the fruit beverages as optimum pH for the growth of most of the food borne pathogens ranges from 6.5 to 7.5. Thus, this potential of enzyme can be manipulated for the preparation of fruit beverages without preservative. In similar findings, optimum pH for α-L- rhamnosidase production from *Aspergillus terreus* and *Aspergillus niger* BCC 25166 was 4 (Abbate *et al* 2012; Petri *et al* 2014; Puri and Banergee 2000; Shamugam and Yadav 1995). Yanai and Sato (2000) reported that enzyme purified from *Pichia angusta* showed optimum activity at pH 6 which is higher than above reported strain. Enzyme production was little affected by pH change in the range 4-6, but yields were low at pH values below 4 (Puri *et al* 2005).

The results presented here demonstrate that among many methods to increase enzyme activity and yield, optimization of medium components and cultivation conditions remains a facile and feasible way to enhance enzyme activity as well as yield.

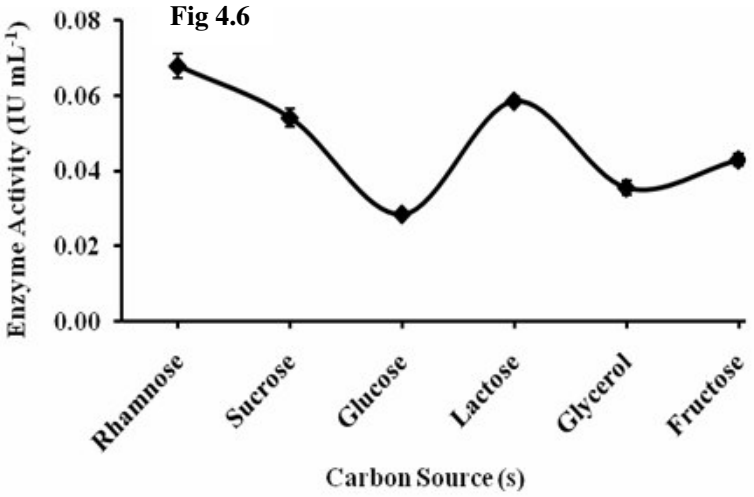


Fig 4.6
Enzyme Activity (IU mL-1)
0.08
0.06
0.04
0.02
0.00
Rhamnose
Sucrose
Glucose
Lactose
Glycerol
Fructose
Carbon Source (s)

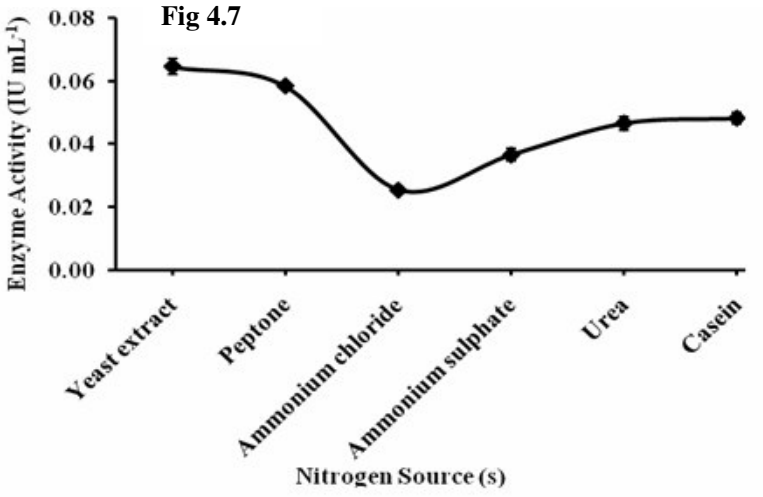


Fig 4.7
Enzyme Activity (IU mL-1)
0.08
0.06
0.04
0.02
0.00
Yeast extract
Peptone
Ammonium chloride
Ammonium sulphate
Urea
Casein
Nitrogen Source (s)

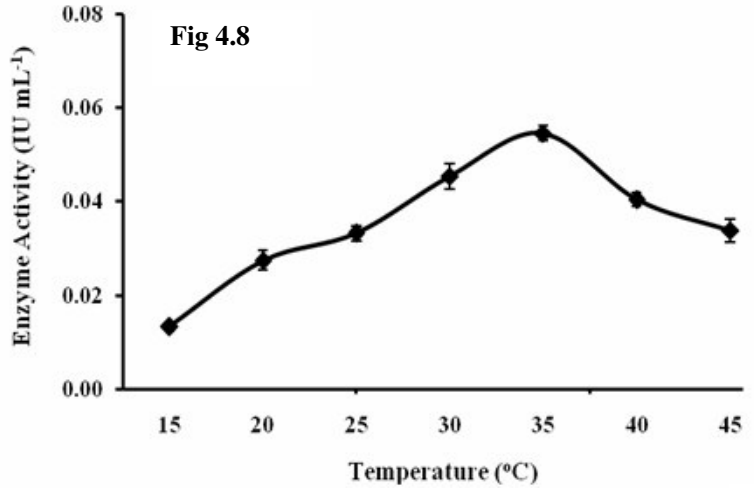


Fig 4.8
Enzyme Activity (IU mL-1)
0.08
0.06
0.04
0.02
0.00
15
20
25
30
35
40
45
Temperature (°C)

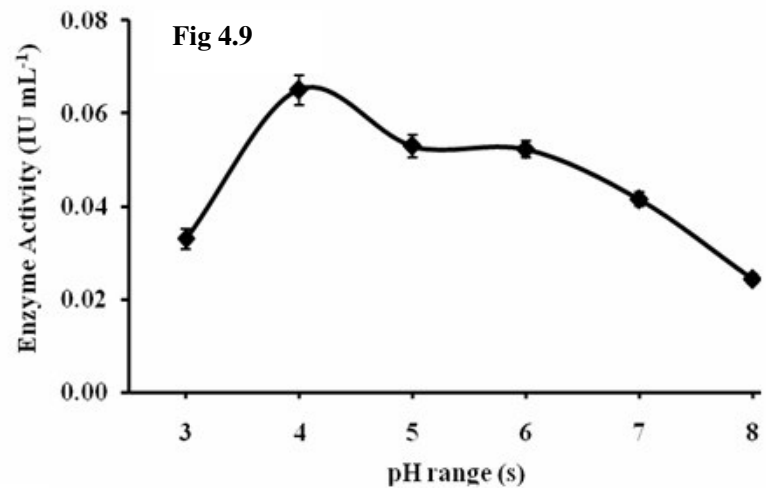
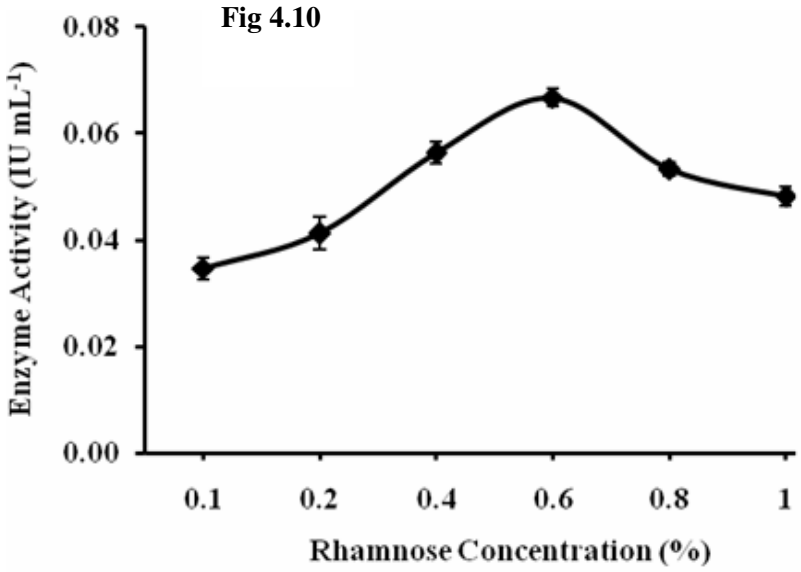


Fig 4.9
Enzyme Activity (IU mL-1)
0.08
0.06
0.04
0.02
0.00
3
4
5
6
7
8
pH range (s)



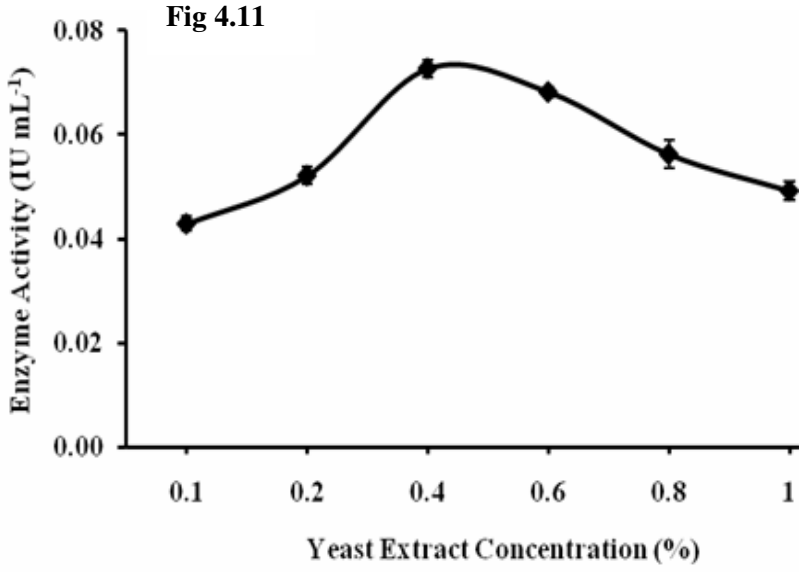


Fig. 4.6 Effect of carbon source on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Fig. 4.7 Effect of nitrogen source on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Fig. 4.8 Effect of temperature on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Fig. 4.9 Effect of pH on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Fig. 4.10 Effect of rhamnose concentration on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Fig. 4.11 Effect of yeast extract concentration on α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446

Table 4.5: Optimization of rhamnose for rhamnosidase production

Rhamnose concentration (%)	Enzyme activity (IU mL^{-1})
0.1	0.036±0.00
0.2	0.037±0.00
0.4	0.058±0.00
0.6	0.065±0.00
0.8	0.054±0.00
1	0.047±0.00

Mean values ± standard error of three independent experiments

Table 4.6: Optimization of yeast extract for rhamnosidase production

Yeast extract concentration (%)	Enzyme activity (IU mL^{-1})
0.1	0.052±0.00
0.2	0.063±0.00
0.4	0.072±0.00
0.6	0.058±0.00
0.8	0.057±0.00
1	0.051±0.00

Mean values ± standard error of three independent experiments

4.8.2 Screening of juice components for optimization of α-L-rhamnosidase production

4.8.2.1 Effect of percent inoculum concentration on α-L-rhamnosidase production

Five different concentrations of standard stock inoculums; 0.25%, 0.5%, 0.75%, 1% and 1.25% (v/v) were added in the juice and incubated for 24 h at room temperature. A differential response in rhamnosidase activity was obtained which showed that the 0.75% inoculum concentration exhibited maximum enzyme activity i.e. 0.057 IU mL^{-1} and 0.25 % exhibited minimum rhamnosidase activity i.e. 0.023 IU mL^{-1} in kinnow juice **(Fig. 4.12)**. Increase in inoculum size resulted in lesser enzyme production, due to the nutrient exhaustion and oxygen limitation. Similar results were also reported using *Bacillus methylotrophicus* (Mukund *et al* 2014) and *Staphylococcus xylosus* MAK2 (Puri and Kalra 2005) for naringinase production. Puri *et al* 2005 studied the inoculum level of 3-15% (v/v) in the salt medium with naringenin as an inducer to establish the effect of inoculum size on the naringinase production by *A. niger*. They observed that 10% (v/v) inoculum was optimal for growth as well as naringinase production and the lag phase was also minimal.

4.8.2.2 Effect of brix (°B) on α-L-rhamnosidase production

In juice, brix was adjusted to 12, 13, 14, 15 and 16 °B by adding sugar solution followed by inoculation of yeast i.e. 0.75% (v/v). It was incubated for 24 h at room temperature. The effect of different °B on yeast rhamnosidase activity was tested and best °B for maximum rhamnosidase activity (0.05 $IUmL^{-1}$) was 13 in juice **(Fig. 4.13)**. Further, with the increase in the initial sucrose concentration the rhamnosidase production was decreased which indicated that the higher sucrose concentration had an adverse effect on the enzyme production efficiency of the yeast. Naringinase activity was repressed by glucose, sucrose, citrate and lactose although these carbon sources supported excellent growth (Puri *et al* 2005; Bram and Solomons 1965). Production of α-L-rhamnosidase by *A. nidulans* is mediated by carbon catabolite repression, which appears to be CreA-independent (Orejas *et al* 1999). Further, it has been reported that the enzyme was not produced when *A. kawachii* was grown on 0.5% glucose as the sole carbon source (Koseki *et al* 2008).

4.8.2.3 Effect of incubation time on α-L-rhamnosidase production

Effect of different incubation time on enzyme activity was studied. Kinnow juice (brix 13 °B and inoculum concentration 0.75% v/v) was incubated for different time periods (12, 24, 36, 48 and 60 h) at room temperature. **Fig. 4.14** shows that the maximum enzyme activity in juice was observed after 48 h of fermentation. In the batch reactor, maximum α-rhamnosidase activity was obtained after 10 days from *Penicillium ulaiense* (Rajal *et al* 2009). The reduction in production time is important because it decreases the fermentation costs and contamination with opportunistic microorganisms during upscale of the process.

4.8.2.4 Effect of temperature on α-L-rhamnosidase production

Kinnow juice (brix 13 °B and inoculum concentration 0.75% v/v) was incubated at different temperatures (15, 20, 25, 30 and 35 °C) for 48 h. The effect of temperature on enzyme activity as Fig. 4.15 shows that the temperature increases, the enzyme activity increased while it decreased at higher temperature at the same time. The decrease in enzyme activity may be due to the deactivation of enzyme due to the weakening of non covalent interactions that stabilize the protein structure. The temperature optima studied is 30±5 °C. The optimum temperature for *Pichia angusta* rhamnosidase was observed at 40 °C (Yanai and Sato 2000). The reported temperature optima of α-L-rhamnosidases are in the range of 40-80 °C though one bacterial α-L-rhamnosidase active at 4 °C is reported (Orrillo *et al* 2007).

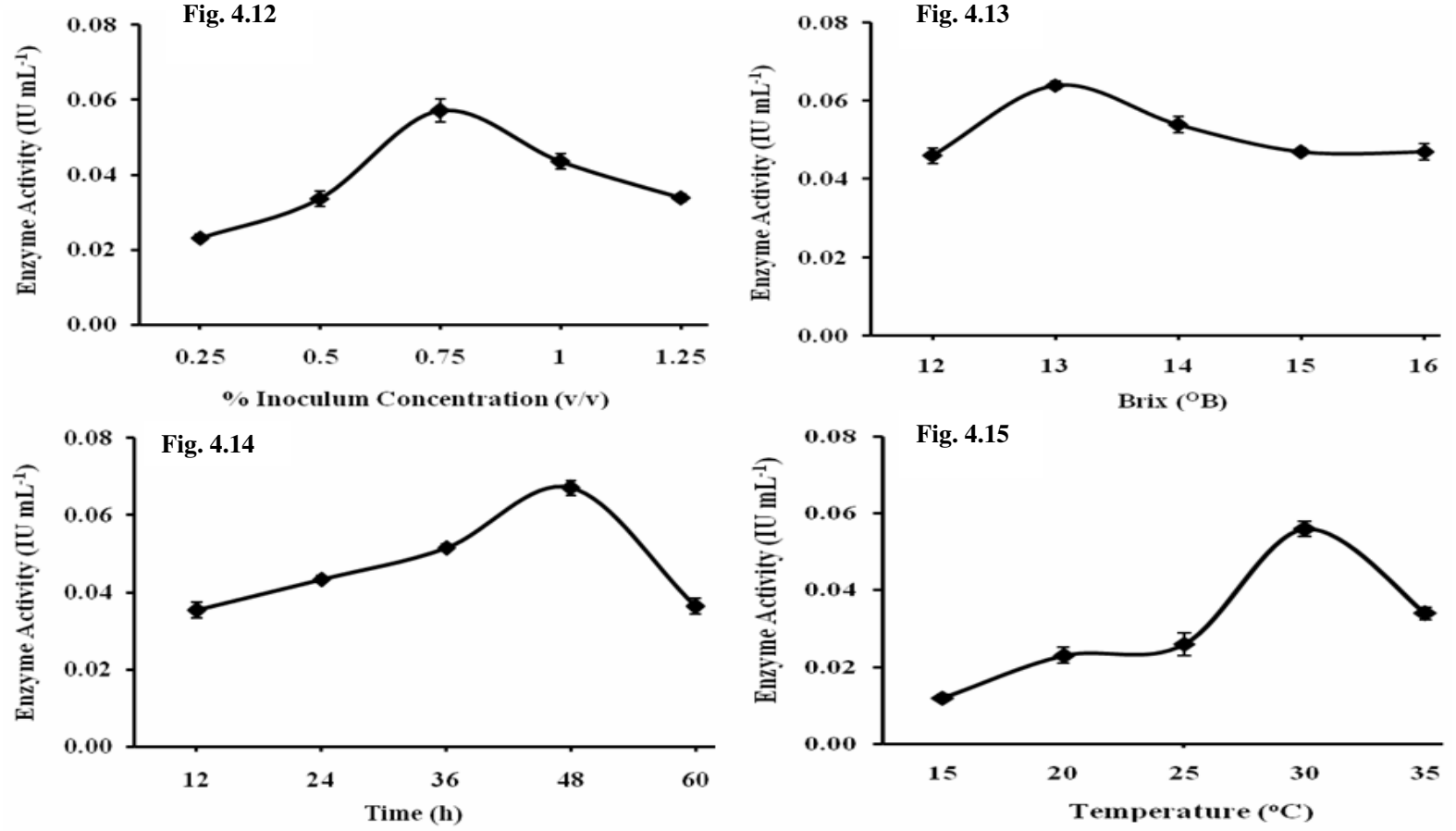


Fig. 4.12 Effect of percent inoculum concentrations on α-L-rhamnosidase production
Fig. 4.13 Effect of brix (°B) on α-L-rhamnosidase production
Fig. 4.14 Effect of incubation time on α-L-rhamnosidase production
Fig. 4.15 Effect of temperature on α-L-rhamnosidase production

4.9 Optimization of screened medium components using Respone Surface Methodology

Following the screening experiments, CCD with 30 experiments was used to determine the optimal levels of the four significant factors (rhamnose, yeast extract, pH and temperature) that affected α-L-rhamnosidase production. The design of experiments and respective experimental and predicted α-L-rhamnosidase activities are given in **Table 4.7**. The results obtained after CCD were analyzed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) of the levels of α-L-rhamnosidase produced (Y) as a function of rhamnose (A), yeast extract (B), pH (C) and temperature (D):

$$Y = 97.28 + 1.29A + 0.708B + 0.54C - 1.29D - 0.68AB - 1.4AC$$

$$Y = + 0.56AD - 1.9BC + 0.18BD + 0.31CD \quad \text{(Equation 1)}$$

$$Y = + 1.3A^2 - 0.69B^2 + 0.427C^2 - 0.9479D^2$$

The significance of the model was also analyzed by analysis of variance (ANOVA) for the experimental design **(Table 4.8)**. Values of "p > F" less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The model F- value of 0.69 implies the model is not significant relative to the noise. There is a 74.89% chance that a F- value this large could occur due to noise. Significant process variables were A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD. The "lack of fit F-value" of 0.58 implies the lack of fit is not significant relative to the pure error. There is a 77.89% chance that a "lack of fit F-value" this large could occur due to noise. The nonsignificant lack of fit of the tested model also indicated that the model was a good fit **(Table 4.8)**.

Low values of coefficient of variation (5.77%) indicate that experimental data were precise and reliable. The goodness of fit of the model was also checked by the coefficient of determination, R^2, which was calculated to be 0.4094. This implies that 40.9479% of experimental data of the α-L-rhamnosidase activity was compatible with the data predicted by the model and only 59.06% of the total variations were not explained by the model. The R^2 value is always between 0 and 1, and a value greater than 0.75 indicates aptness of the model. For a good statistical model, R^2 value should be close to 1.0. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The results 4.001 indicate an adequate signal and this model can be used to navigate the design space. A negative predicted R^2 (-1.689) implies that the overall mean is a better predictor of the response than the current model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted R^2 was -0.18. All these considerations indicate

Table 4.7: Design of RSM experiments and respective experimental and predicted α-L-rhamnosidase activities

Variables under study				α-L-rhamnosidase activity (IU L^{-1})	
Rhamnose (g L^{-1})	**Yeast Extract (g L^{-1})**	**pH**	**Temperature (°C)**	**Experimental value**	**Predicted Value**
0.1	0.1	3	30	99	96
0.6	0.1	3	30	103	102
0.1	0.6	3	30	97	109
0.6	0.6	3	30	98	93
0.1	0.1	5	30	97	101
0.6	0.1	5	30	106	104
0.1	0.6	5	30	97	99
0.6	0.6	5	30	100	106
0.1	0.1	3	40	90	92
0.6	0.1	3	40	109	91
0.1	0.6	3	40	106	98
0.6	0.6	3	40	106	102
0.1	0.1	5	40	103	101
0.6	0.1	5	40	95	103
0.1	0.6	5	40	105	95
0.6	0.6	5	40	110	99
0.35	0.35	4	35	90	109
0.35	0.35	4	35	106	103
0.35	0.35	4	35	92	92
0.35	0.35	4	35	90	95
-0.15	0.35	4	35	102	95
0.85	0.35	4	35	110	106
0.35	-0.15	4	35	110	91
0.35	0.85	4	35	96	94
0.35	0.35	2	35	109	100
0.35	0.35	6	35	96	94
0.35	0.35	4	25	103	92
0.35	0.35	4	45	109	91
0.35	0.35	4	35	110	96
0.35	0.35	4	35	91	93

Table 4.8: ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F Value	*p*-value Prob > F	
Block	123.2667	1	123.2666667			
Model	311.45	14	22.24642857	0.693421	0.74889	Non significant
A-Rhamnose	40.04167	1	40.04166667	1.248098	0.282722	
B-Yeast Extract	12.04167	1	12.04166667	0.375339	0.549931	
C-pH	7.041667	1	7.041666667	0.219489	0.646641	
D-Temperature	40.04167	1	40.04166667	1.248098	0.282722	
AB	7.5625	1	7.5625	0.235723	0.634822	
AC	33.0625	1	33.0625	1.030558	0.327252	
AD	5.0625	1	5.0625	0.157798	0.697182	
BC	60.0625	1	60.0625	1.872147	0.192789	
BD	0.5625	1	0.5625	0.017533	0.896542	
CD	1.5625	1	1.5625	0.048703	0.828522	
A^2	46.50298	1	46.50297619	1.449497	0.24857	
B^2	13.36012	1	13.36011905	0.416435	0.529156	
C^2	5.002976	1	5.00297619	0.155943	0.698871	
D^2	24.64583	1	24.64583333	0.76821	0.395562	
Residual	449.15	14	32.08214286			
Lack of Fit	265.9	10	26.59	0.580409	0.778944	Not significant
Pure Error	183.25	4	45.8125			
Cor Total	883.8667	29				

AB, AC, AD, BC, BD and CD represent the interaction effect of variables A, B, C and D; A^2, B^2, C^2 and D^2 are the square effects of the variables

Table 4.9: Model Fitting Values of RSM

Model terms	Values
Standard deviation	5.66411
Mean	98.06667
Coffecient of variation (%)	5.775775
PRESS	2045.362
R^2	0.409479
Adjusted R^2	-0.18104
Predicted R^2	-1.68914
Adequate precision	4.000985

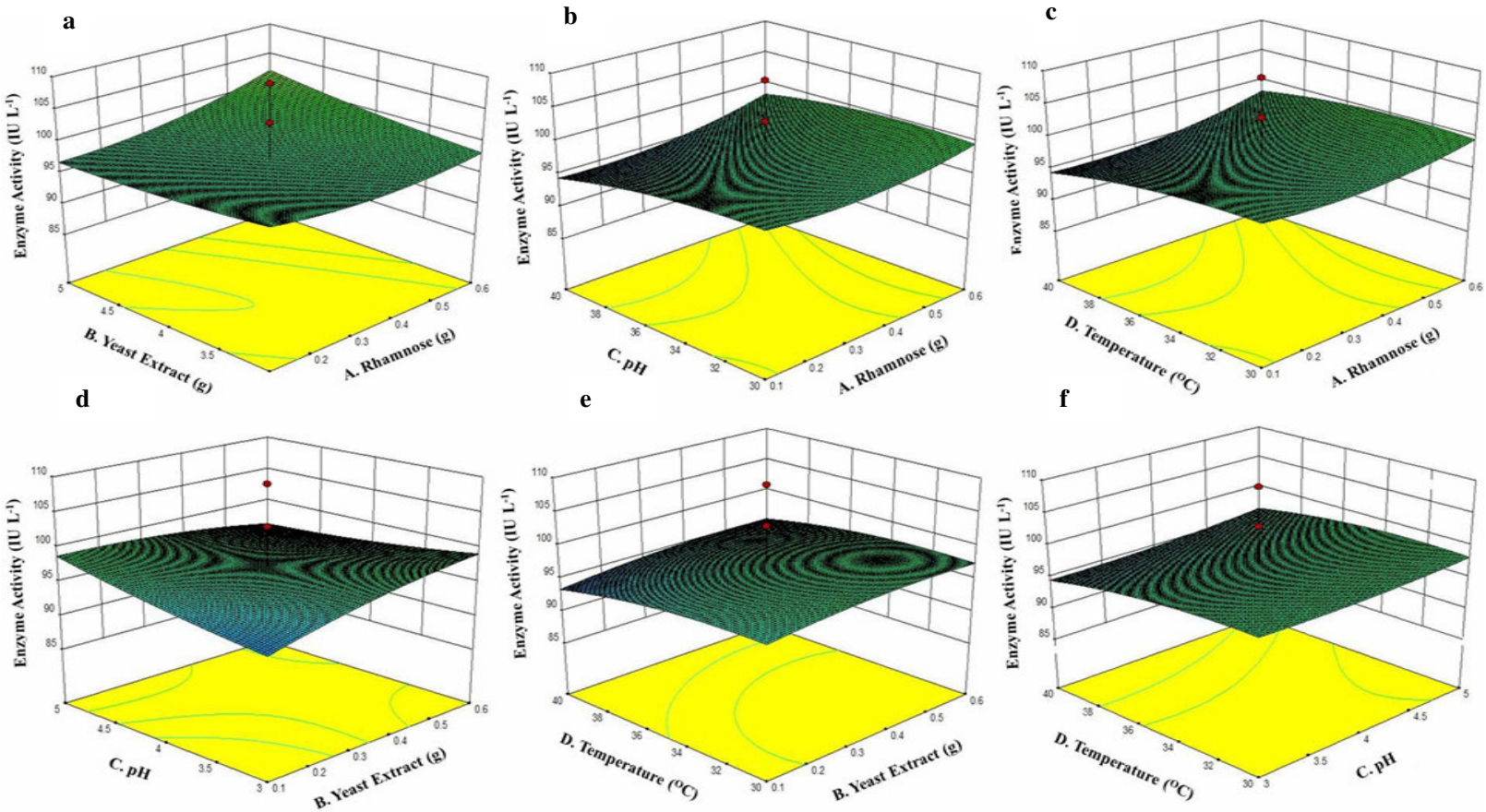


Fig. 4.16 Three-dimensional response surface plot of α-L-rhamnosidase production by *Clavispora lusitaniae* KF633446 showing the interaction between (a) yeast extract and rhamnose (b) pH and rhamnose (c) temperature and rhamnose (d) pH and yeast extract (e) temperature and yeast extract and (f) temperature and pH on α-L-rhamnosidase production (IU L^{-1})

good adequacy of the regression model **(Table 4.9)**.

The three-dimensional response surface and contour plots described by the regression model are presented in **Fig. 4.16**. These plots were obtained from the pair wise combination of two independent variables, while keeping the other two variables at their center-point levels. From the curve of three-dimensional plots, optimal composition of medium components can be identified. The contour plots highlight the roles played by the process variables (rhamnose, yeast extract, pH and temperature) and their interactive effects. From **Fig. 4.16** it is evident that increase in concentration of variables had a positive influence on α-L-rhamnosidase activity until an optimum value was reached, beyond which variables had significant negative influence on the α-L-rhamnosidase activity. The contour plots show a rather broad plateau region in which the activities change relatively little when the nutrient concentrations were varied. This indicates that the optimal solution can accommodate small errors or variability in the experimental factors.

Mukund *et al* (2014) applied response surface modeling based on central composite design to determine the effects of three independent variables (sucrose, yeast extract and naringin) and their mutual interactions. In total, 20 experiments were conducted and a statistical model was developed, which predicted naringinase production of 10.61 U L^{-1}. Subsequently, verification experiments were conducted and validity of the model was verified. Bioreactor studies conducted with the optimized medium showed an enzyme production of 12.05U L^{-1} within 34 h of fermentation (Mukund *et al* 2014). Response surface methodology has been successfully applied for the quantitative study of enzyme stability. Compared to the traditional approach consisting of changing one variable at a time, response surface methodology significantly reduced experimental effort and facilitated data treatment and interpretation of the results. Thus, temperature, pH and enzyme concentration effect on stability were evaluated on a minimum set of optimal selected experiments (Magario *et al* 2009).

4.10 Purification of α-L-rhamnosidase

Enzyme α-L-rhamnosidase enzyme was purified from the culture filtrate of *Clavispora lusitaniae* KF633446 grown on L-rhamnose as sole carbon source using anion exchange chromatography. The results of purification procedure are summarized in **Table 4.10**. Elution profile of the enzyme from the DEAE sephadex A-50 column is presented in **Fig. 4.17**, showing maximum rhamnosidase activity in fraction number 20 to 33. The purification steps were combined to give an overall purification of about 10.1-fold with 46.98% recovery of rhamnosidase activity. The total activity of purified enzyme was 24.9 IU and specific activity was 2.7 IU mg^{-1}. An enzyme showing α-L-rhamnosidase activity was purified to apparent homogeneity from the culture filtrate of *A.nidulans* grown on L-rhamnose as sole carbon source using ion exchange and gel filtration chromatographies, resulted in a

level of recovery of 6.3% of the original α-L-rhamnosidase activity and 18-fold increase in specific activity (Manzanares *et al* 2000). α-L-Rhamnosidase was purified 662-fold with a recovery of 8.03% from *Bacillus* sp. GL1 cells grown in the gellan medium (Hashimoto *et al* 1999), 330-fold with a recovery of 19.5% from the crude cell extracts of *P. paucimobilis* FP2001 (Miake *et al* 2000) and 18-fold purification with 25% recovery in the medium amended with 0.5% naringin (Yadav *et al* 2012).

Table 4.10: Summary of purification procedure of α-L-rhamnosidase from *Clavispora lusitaniae* KF633446

Purification step	Volume (mL)	Activity (IU)	Protein (mg)	Specific activity (IU mg^{-1})	Yield (%)	Fold purification
Culture filtrate	500	53	206	0.25	100	1
Ammonium sulphate precipitation	38	49.5	110	0.45	93.39	1.8
Dialysis	7	46.7	69.4	0.67	88.11	2.68
DEAE sephadex A-50 chromatography and dialysis	5	24.9	9.2	2.70	46.98	10.8

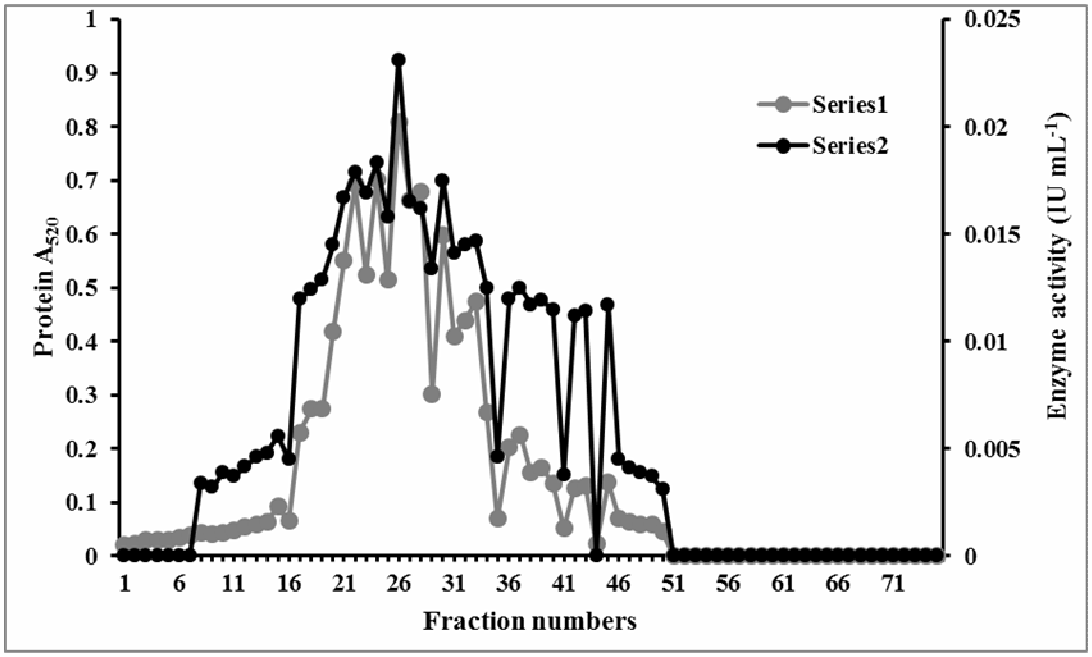


Fig 4.17 Elution profile of *Clavispora lusitaniae* α-L- rhamnosidase on DEAE-Sephdex A-50

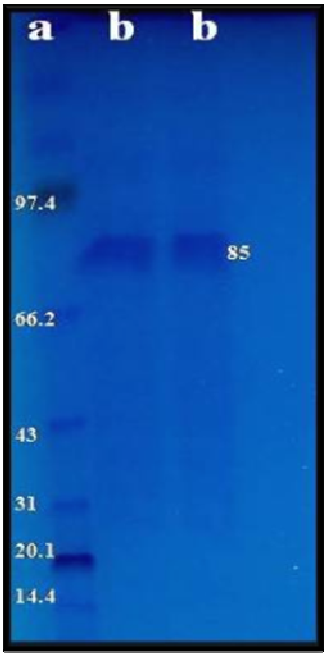


Plate 4.7 Results of SDS-PAGE analysis of the purified enzyme (a) lane 1 phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4kDa) (b) lane 2 and 3 (purified enzyme)

Plate 4.8 Lyophilized purified α-L-rhamnosidase enzyme from *Clavispora lusitaniae* KF633446

The result of SDS-PAGE analysis revealed a single protein band with an apparent molecular weight of 85 kDa **(Plate 4.7 and Plate 4.8)**. The relative molecular masses of the reported α-L-rhamnosidases are in the range 53.0–240.0 kDa though in some cases oligomeric forms of the enzyme having relative molecular mass as high as 500 kDa have been reported (Kim *et al* 2005). The value obtained was similar to the molecular mass of extracellular α-L-rhamosidase from *Aspergillus niger* (Manzanares *et al* 1997) and *Aspergillus aculeatus* (Manzanares *et al* 2001) and closer to the molecular mass of *Aspergillus aculeatus* (87 kDa) (Mutter *et al* 1994), *Aspergillus niger* (87 kDa) (Ni *et al* 2012), *Pichia angusta* (90 kDa) (Yanai and Sato 2000) and *Aspergillus kawachii* (90 kDa) (Koseki *et al* 2008).

4.11 Enzyme characteristics

4.11.1 Effect of pH on the activity of purified α-L-rhamnosidase

The activity of α-L-rhamnosidase enzyme at different pH values is shown in **Fig. 4.18**. The purified enzyme was most active at pH 4 using p-nitrophenyl-α-L-rhamnopyranoside as the substrate B, which is close to that of fruit juice, making it suitable for use in fruit juices and wine. Broad pH optima are preferable for food applications and most of the α-L-rhamnosidases reported so far have pH optima in the range 2.0-8.0. The purified enzyme having optimum pH in the neutral pH range is more suitable for the enhancement of wine aroma (Yadav *et al* 2012). The α-L-rhamnosidases reported from *Aspergillus aculeatus* (Manzanares *et al* 2001), *Curvularia lunata* (Feng *et al* 2007), *Aspergillus niger* (Puri and Kalra 2005) and *Aspergillus kawachii* (Koseki *et al* 2008) have same optimum pH i.e. 4. Yanai and Sato (2000) reported that the enzyme from yeast *Pichia angusta* showed optimum activity at pH 6. *Penicillium paucimobilis* FP2001 and *Sphingomonas sp.* R1 (Hashimoto and Murata 1998) Rhase had pH optimums at slightly alkaline pH.

4.11.2 Effect of temperature on the activity of purified α-L-rhamnosidase

The optimum temperature of the purified enzyme is 50 °C **(Fig. 4.19)**. The optimum temperature obtained was similar to the α-L-rhamnosidases from *Bacillus sp* GL1 (Hashimoto *et al* 1999), *Lactobacillus plantarum* NCC 245 (Avila *et al* 2009), *Aspergillus flavus* (Scaroni *et al* 2002), *Curvularia lunata* (Feng *et al* 2007), *Aspergillus niger* (Puri and Kalra 2005) and *Aspergillus kawachii* (Koseki *et al* 2008). Enzymes from *Pichia angusta* (Yanai and Sato 2000), *Aspergillus kawachii*, *Penicillium aureatiogriseum* and *Trichoderma longibrachiatu* (Scaroni *et al* 2002) were optimally active at 40 °C and 60 °C. This phenomenon suggested that the temperature for enzymatic hydrolysis of naringin and conversion of other flavonoids should be controlled under 50 °C.

4.11.3 Thermal stability of purified enzyme

The thermal stability result of the purified enzyme is shown in **Fig. 4.20**. The enzyme was relatively stable up to 50 °C but temperature higher than 60 °C resulted in a rapid loss of

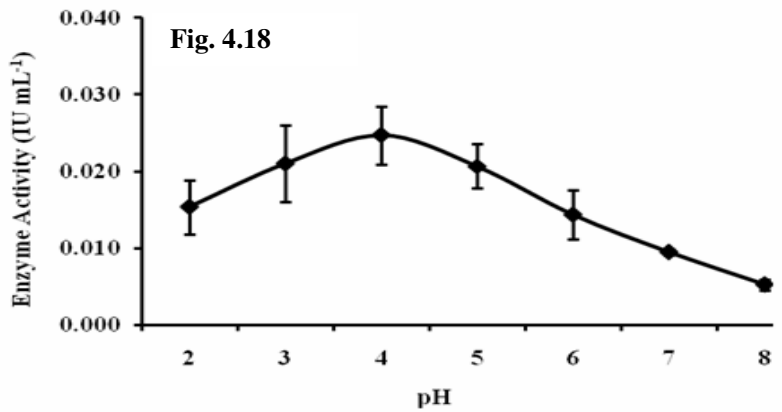
activity. Thermal stability of the α-L-rhamnosidase is an important criterion for its application in industrial processes. According to Yadav *et al* (2012), the enzyme from *Penicillium citrinum* is stable at 20 °C but looses activity very rapidly above 60 °C. The α-L-rhamnosidase produced by *Acrostalagmus luteo albus* was stable up to 40 °C for 4 h, whereas it retained 95% of its original activity after 10 h of incubation, even when no protective agent was added (Rojas *et al* 2011). The activity and stability shown by α-L-rhamnosidase at higher temperatures are more than other reported yeast strains, which undergo inactivation at higher temperatures. Thus, the high activity and stability over broad temperature range suggest its usefulness in industrial applications.

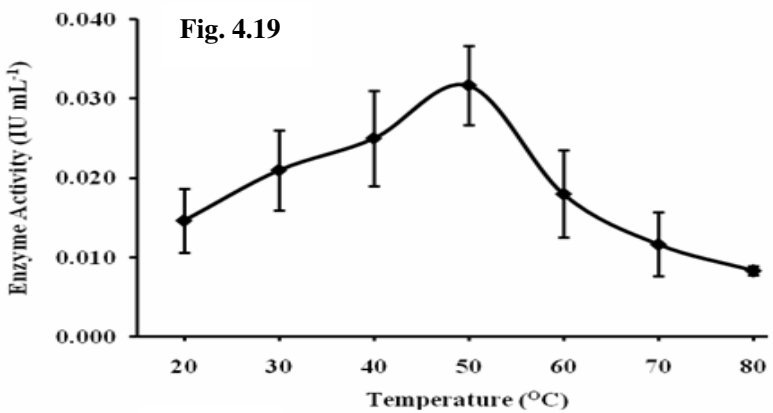
4.11.4 Effect of metal ions on the activity of purified enzyme

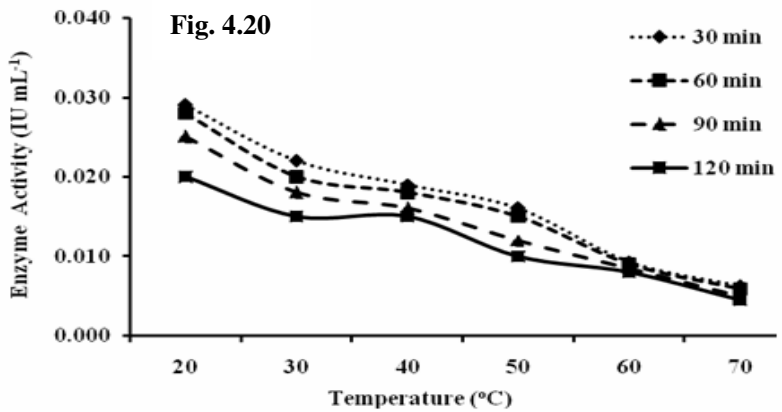
The effects of various metal ions (1mM) on the activity of purified enzyme were determined and results in **Fig. 4.21** and **Table 4.11** shows enzyme activity strongly inhibited by Ag^{+} and slightly inhibited by Mg^{++}, Cu^{++} and Zn^{++} cations. However, other cations Mn^{++}, Ca^{++}, Co^{++}, K^{+} and Na^{+} slightly activated the enzyme activity. Yadav *et al* (2012) reported that the enzyme activity was inhibited by 0.5 mM Zn^{++} and Cu^{++} and activated by Mn^{++} and Ca^{++} ions. In contrast, 0.5 mM Co^{++}, K^{+} and Na^{+} ions inhibited the enzyme activity (Yadav *et al* 2012). Thus the rhamosidase activity in citrus juices can also be affected by metal ions because citrus fruits contain minerals: calcium, copper, iron, magnesium, manganese and zinc.

Table 4.11: Effect of metal ions on the activity of purified enzyme

Sr. No.	Metal ions (1mM)	Residual activity (%)
1	Control	100
2	$MnSO_4$	113
3	$CuSO_4$	47
4	$CaCl_2$	107
5	KCl	120
6	$ZnSO_4$	76
7	NaCl	106
8	$CoCl_2$	101
9	AgCl	5
10	$MgCl_2$	82







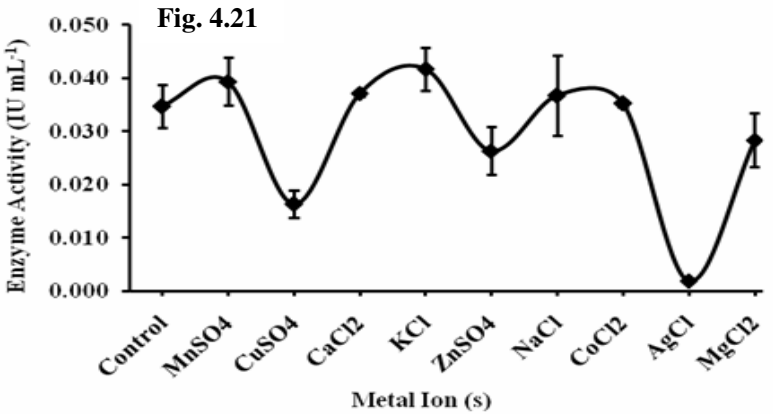


Fig. 4.18 Effect of pH on the activity of purified enzyme

Fig. 4.19 Effect of temperature on the activity of purified enzyme

Fig. 4.20 Thermal stability of purified enzyme

Fig. 4.21 Effect of metal ions on the activity of purified enzyme

4.11.5 Steady-state enzyme kinetics

The Km and Vmax of purified enzyme using p-nitrophenyl α-L-rhamnopyranoside as the substrate were calculated from the double reciprocal plots to be 0.18 mM and 25 IUmL^{-1} respectively **(Fig. 4.22)**. The reported Km values for p-nitrophenyl-α-L-rhamnopyranoside are in the range 0.057-2.8 mM. The Km and Vmax values of the enzyme were 0.36 mm and 22.54 µmole min^{-1} mg^{-1} for *Penicillium citrinum* (Yadav *et al* 2012) and 0.27 mmol L^{-1} and 64.6 U mg^{-1} for *Aspergillus nidulans* (Manzanares *et al* 2000). Rojas *et al* (2011) reported that the Km and Vmax values for α-L-rhamnosidase purified from *Acrostalagmus luteo albus* were 3.38 mmol L^{-1} and 68.5 mmol L^{-1} min^{-1}, respectively.

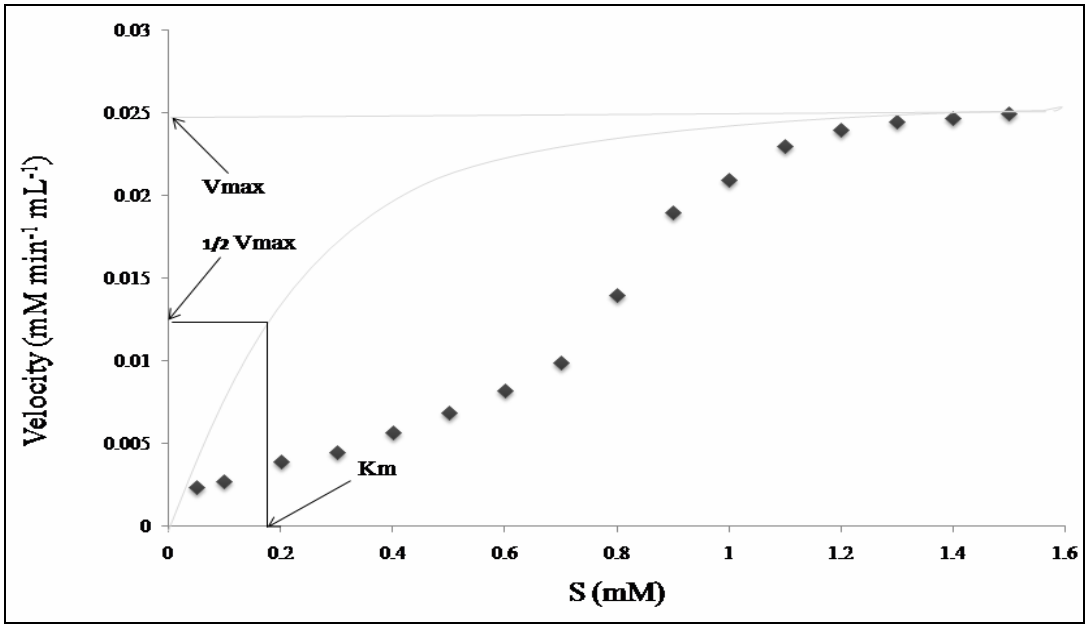


Fig. 4.22 Double reciprocal plots of the purified enzyme using p-nitrophenyl α-L-rhamnopyranoside as the substrate

4.12 Test for food grade enzyme

4.12.1 Oral acute toxicity of concentrated purified α-L- rhamnosidase enzyme

Enzyme α-L- Rhamnosidase is an edible food grade enzyme so animal study was conducted. All mice treated with and without the enzyme powder (0-0.5 mg/g body weight) survived during the 10-d observation period. All the mice showed normal physiology and behavior during the administration. Neither the symptoms of toxicity nor the significant differences in growth (body weight) and food intake were observed **(Table 4.12 and Plate 4.9)**. Thus the results showed that the purified enzyme was nontoxic and safe for food use. Similar results have been reported by Ni *et al* (2012) revealed the maximum tolerated dose (MTD) of the naringinase powder from *Aspergillus niger* was >10 g kg^{-1} in mice.

Plate 4.9 Sweet albino male mice used in oral acute toxicity experiment

Table 4.12: Effect of maximum tolerance dose (MTD) of enzyme on mice

Groups	Rhamnosidase enzyme dose (mg/g BW)	Average weight of mice after administration (g)	Average food consumption in the last 5 days of administration [g/(mice.d)]
Group I	0.5	29.65±0.482	7.67±0.225
Group II	1	28.66±0.971	7.55±0.240
Control	0	30.37±1.052	7.66±0.104

*BW- Body weight, Mean values ± standard error of three independent experiments

4.12.2 Metals analysis of purified enzyme

The heavy metals As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in the enzyme powder were observed in range of 0.003 mg Kg^{-1}, 0.001 mg Kg^{-1}, 0.105 mg Kg^{-1}, 0.285 mg Kg^{-1}, 3.162 mg Kg^{-1}, 0.109 mg Kg^{-1}, 0.067 mg Kg^{-1}, 0.011 mg Kg^{-1} and 0.391 mg Kg^{-1} and these are much below than the permitted maximum levels for food additives **(Table 4.13)**. The below Joint FAO/World Health Organization Expert Committee on Food Additives (JECFA) has given guidelines as provisional tolerable weekly intake (PTWI) for lead, arsenic and cadmium as 0.025 mg Kg^{-1}, 0.015 mg Kg^{-1} and 0.007 mg Kg^{-1} body weight (JECFA 2004). The FAO/WHO set up the upper limits of arsenic, cadmium, copper, lead and zinc on 0.5 mg Kg^{-1}, 0.5 mg Kg^{-1}, 2 mg Kg^{-1}, 2 mg Kg^{-1} and 0.5 mg Kg^{-1}, respectively (FAO/WHO 2011). So the results suggest the enzyme fulfills the requirements of safety for commercial use.

Table 4.13: Heavy metal analysis of purified α-L-rhamnosidase enzyme

Sr.No.	Heavy metals	mg Kg^{-1}	Permissible limit
1	Arsenic	0.003	0.015 mg Kg^{-1} (JECFA 2004) and 0.5 mg Kg^{-1} (FAO/WHO 2011)
2	Cadmium	0.001	0.007 mg Kg^{-1} (JECFA 2004) and 0.5 mg Kg^{-1} (FAO/WHO 2011)
3	Chromium	0.105	-
4	Copper	0.285	2 mg Kg^{-1} (FAO/WHO 2011)
5	Iron	3.162	-
6	Manganese	0.109	-
7	Nickel	0.067	-
8	Lead	0.011	0.025 mg Kg^{-1} (JECFA 2004) and 2 mg Kg^{-1} (FAO/WHO 2011)
9	Zinc	0.391	0.5 mg Kg^{-1} (FAO/WHO 2011)

4.13 Debittering of juice

4.13.1 Physicochemical characteristics of Kinnow *(Citrus reticulate* Blanco)

The physicochemical composition of fresh kinnow juice was evaluated on the basis of chemical analysis, **(Table 4.14)** as TSS- 8±1 °B, titrable acidity- 0.29±0.02%, pH- 3.5±0.1, brix acid ratio- 27.58±1, total sugars- 8.9±0.1%, reducing sugars- 2±0.5%, ascorbic acid- 30.4±1 mg/100 g, total polyphenol content- 58.4±2 mg GAE/100 mL, limonin- 7.4±0.2 ppm, naringin- 421.8 ± 10 ppm, juice yield- 55±5% and peel and pomace- 39±4% **(Plate 4.10)**. In kinnow fruit, the juice content was found to be in the range of 36% to 62% (Jagjiwan 2001) and ascorbic acid content in the range of 13.3 to 46.9 mg/100 mL (Pruthi *et al* 1983; Singh *et al* 1978). The acidity, pH and TSS of kinnow juice were reported in the range of 0.28-0.51%, 4.20-4.28 and 8-15.75% by Kaur 2002. The amount of reducing and nonreducing sugars has been found around 3.95% and 3.65% (Veldihus 1971). The acceptability and higher sensory score of beverages is very much dependent on its physicochemical properties including appearance, flavor, acidity and TSS. There may be changes in the physicochemical characteristics and loss of some compounds that impart flavour and aroma to the beverages during pasteurization and storage (Jairath *et al* 2012). The juice obtained was further debittered by two process (juice+enzyme) and (juice+*Clavispora lusitaniae*) to produce low alcoholic naturally carbonated beverage.

Table 4.14: Physicochemical characteristics of kinnow (*Citrus reticulata* Blanco)

Parameters	Kinnow juice
TSS (°B)	8±1
Acidity (%)	0.29±0.02
pH	3.5±0.1
Brix-acid ratio	27.58±1
Total sugars (%)	8.9±0.1
Reducing sugars (%)	2±0.5
Ascorbic acid (mg/100 mL)	30.4±1
Total polyphenol content (mg GAE/100 mL)	58.4±2
Limonin (ppm)	7.4±0.2
Naringin (ppm)	421.8±10
Juice yield (%)	55±5

Mean values ± standard error of three independent experiments

Plate 4.10 Kinnow (*Citrus reticulata* Blanco)

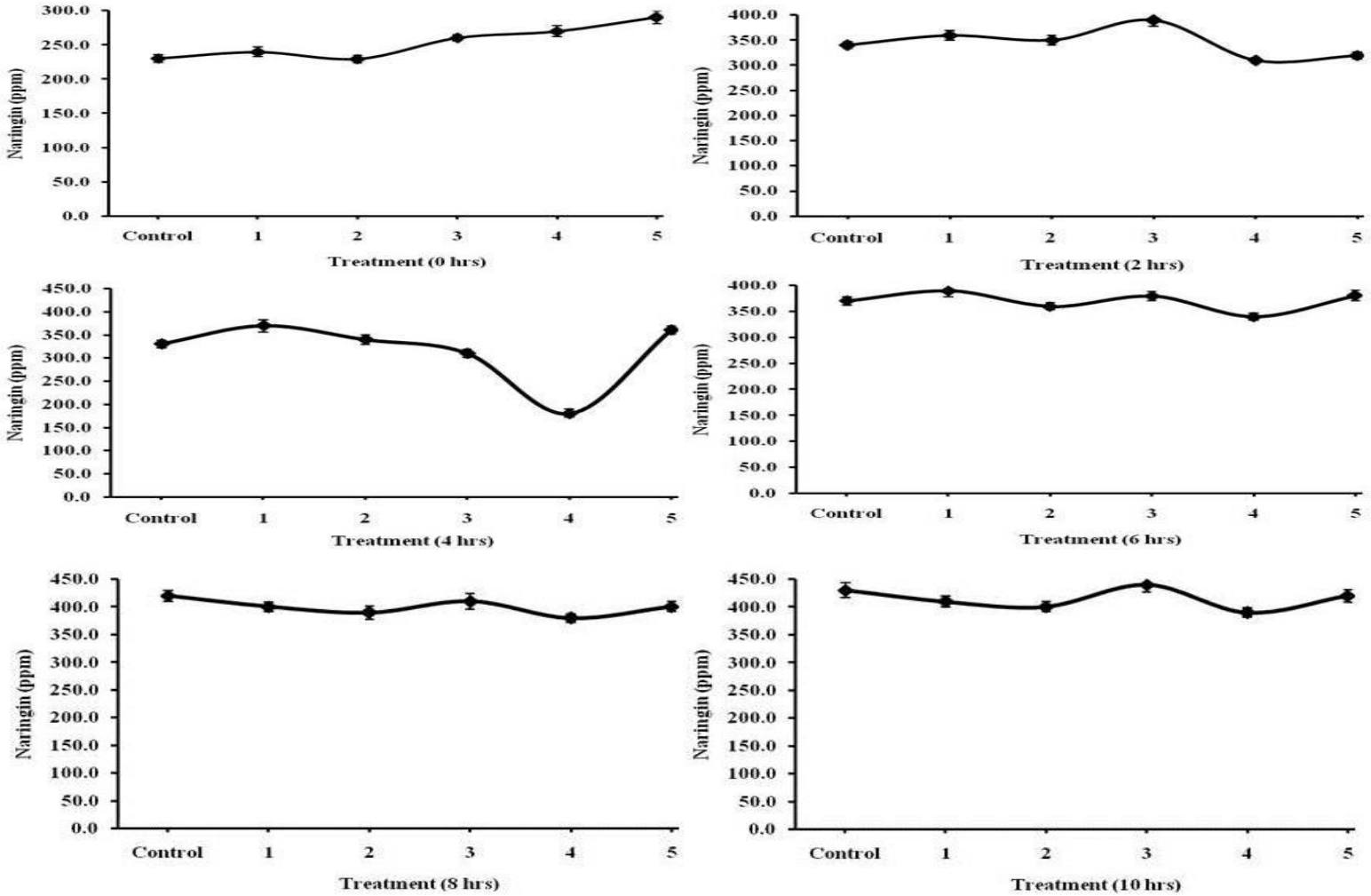
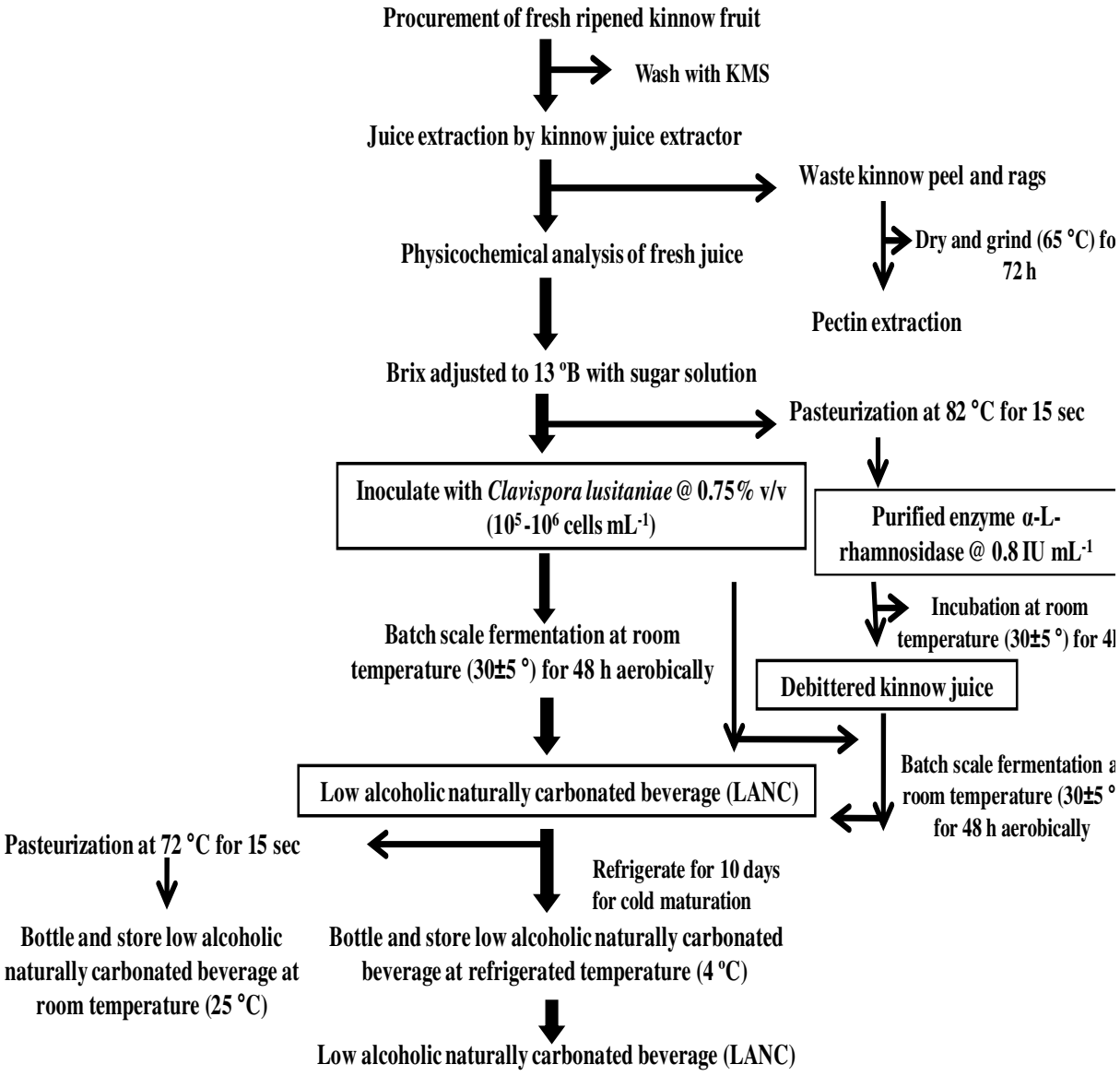


Fig. 4.23 Optimizing the concentration and time of incubation of purified α-L-rhamnosidase enzyme in debittering of kinnow juice



(TSS 11.2 °B, pH 3.0, acidity 0.56%, total sugars 9.5%, reducing sugars 1.32%, ascorbic acid 6.48 mg/100 mL, limonin 3.52 ppm, naringin 143.7 ppm, alcohol 0.89 % and CO_2 pressure 1.46 bar)

Flow diagram: Production of debittered kinnow juice and fermented low alcoholic naturally carbonated beverage (Jairath and Sahota 2011) using purified α-L-rhamnosidase enzyme and *Clavispora lusitaniae*, respectively

Plate 4.11 Kinnow juice containing different concentration of enzyme (0.2-1 IU mL^{-1})

Plate 4.12 Large scale production of debittered kinnow juice

4.12.2 Preparation of debittered kinnow juice

Trials were conducted to debitter kinnow juice by using enzyme (0.2-1.0 IU mL^{-1}) in diluted juice (13 °B) **(Fig. 4.23 and Plate 4.11)**. Results showed that the maximum reduction in naringin concentration i.e. 180.71 ppm was observed using enzyme activity 0.8 IU mL^{-1}, in time period of 4 h suggesting our enzyme is efficient in reducing the bitterness in kinnow juice. The optimized conditions (enzyme 0.8 IU mL^{-1} and incubation time 4 h) were used for large scale production of debittered kinnow juice **(Table 4.15 and Plate 4.12)**. The presence of naringin in excess 600 ppm has been established as an objectionable level of bitterness in processed citrus products such as juice, wine and vinegar (Guadagni *et al* 1973). Maximum decrease of naringin (about 74%) was obtained at naringinase concentration of 100 U/100 mL grapefruit juice, with incubation at 40 °C for 4 h (Patil and Dhake 2014).

Table 4.15: Optimized parameters for debittering of kinnow juice and beverage

Kinnow juice		**Low alcoholic naturally carbonated kinnow beverage**	
Enzyme activity	0.8 IU mL^{-1}	Yeast inoculum concentration	0.75 % (v/v)
TSS	13 °B	TSS	13 °B
Temperature	30±5 °C	Temperature	30±5 °C
Incubation time	4 h	Incubation time	48 h

4.12.3 Upscale production of fermented debittered kinnow beverage

The optimized process parameters for preparation of fermented debittered kinnow beverage from *Clavispora lusitaniae* from 4.8.2 were kinnow juice 40%, degree brix 13 °B, inoculum concentration 0.75% v/v, temperature 30±5 °C and incubation time 48 h. The experiment was conducted in 10 L fermenter **(Table 4.15, Plate 4.13, Plate 4.14 and Plate 4.15)**.

The formulation of naturally carbonated beverage offers more variety of flavours, nutrients, long shelf life and other physiological benefits with a greater margin of safety in a drink with a lower inherent cost as compared to fruit juice. The fermented beverage retains nutrients and the additional CO_2 so produced is antimicrobial and adds tangy taste, fizz and sparkle to the beverage (Jairath *et al* 2012, Sahota *et al* 2009). CO_2 is an antimicrobial agent itself acting as a critical solvent and week organic acid (upon dissolution) penetrating plasma membrane and acidifying cell anterior of microorganism, thus responsible for enhancing shelf life of the beverage. The effectiveness of carbonation is the main factor in determining the quality and consumer acceptance of final beverage.

4.12.3.1 Shelf life studies of kinnow beverage

Shelf life of fermented debittered kinnow beverage stored at refrigerated temperature (4 °C) and room temperature was studied and evaluated fortnightly for physicochemical, microbiological and organoleptic qualities.

4.12.3.1.1 Evaluation of microbiological and physicochemical properties of kinnow beverage during storage

The results of microbiological and physicochemical properties of kinnow beverage during storage period of 90 days are summarized in **Table 4.16** and **Table 4.17**. The results showed a significant decrease in brix from 13±0.2 °B to 11.2±0.3 °B at refrigerated temperature and significant increase in brix from 13±0.2 °B to 13.95±0.1 °B at room temperature. The brix acid ratio decreased from 92.85±0.0 to 19.31±0.0 at refrigerated temperature and 92.85±0.0 to 23.25±0.0 at room temperature. Similar results have been reported in lime juice (Sarolia and Mukherjee 2002), kinnow sera, cane and kinnow cane juice (Khandelwal *et al* 2006) and ready to serve mandarin (*Citrus reticulata*) diet drink (Ahmed *et al* 2008). The increase in TSS content of juice during storage might be due to hydrolysis of polysaccharides into monosaccharide and oligosaccharides.

The pH also decreased from 3.4±0.1 to 3.0±0.1 at refrigerated temperature and 3.4±0.1 to 3.1±0.3 at room temperature while acidity increased from 0.14±0.03% to 0.56±0.01% at refrigerated temperature and 0.14±0.03% to 0.60±0.03% at room temperature. pH is inversely proportional to the acidity of any medium. This decrease in pH and increase in acidity was attributed to formation of acidic compounds by degradation of reducing sugars (Zia 1987; Akhtar *et al* 2010). Our results are in confirmatory with Saleem (1980) and Ahmed (2008).

The total sugars decreased from 12.9±0.3% to 9.5±0.4% at refrigerated temperature while increase in total sugars from 12.9±0.3% to 13.5±0.3% at room temperature. The reducing sugars also decreased from 2.42±0.2% to 1.32±0.1% at refrigerated temperature while increased from 2.42±0.2% to 2.69±0.2% at room temperature. The sugars in citrus are mainly glucose, laevulose and sucrose. Similar results were also observed by Jairath (2012) for preparation of amla beverage. The increase in total sugar content of juice during storage might be due to hydrolysis of polysaccharides into monosaccharide and oligosaccharides.

Ascorbic acid (vitamin C) content was reduced from the initial concentration 27.8±1 mg/100 mL to 6.48±0.4 mg/100 mL at refrigerated temperature and 27.8±1 mg/100 mL to 5.9±0.5 mg/100 mL at room temperature. The decrease of ascorbic acid (vitamin C) in beverage during storage results from oxidation of ascorbic acid by ascorbic acid oxidase due to a combined effect of oxygen and light (Bhardwaj and Mukherjee 2011; Jairath *et al* 2012; Bhardwaj 2013). But the decrease was lower under refrigerated storage condition which may be attributed to low temperature and high relative humidity in storage, which inhibited the

Plate 4.13 Batch scale fermentation in fermenters

Plate 4.14 Carbonation in glass bottles

Plate 4.15 Large scale production of debittered kinnow beverage

conversion of acid in sugars and decreased rate of ascorbic acid oxidation.

The mean polyphenol contents of kinnow beverage were significantly decreased from 53.48±3 mg GAE/100 mL to 40.8±2 mg GAE/100 mL at refrigerated temperature and 53.48±3 mg GAE/100 mL to 35.4±3 mg GAE/100 mL at room temperature during storage. The polyphenol contents of commercial fruit juices in the case of pineapple, orange and mango juices were higher than those of Thai beverages, reported by Abdullakasim *et al* (2007). Different factors such as processing techniques, clarification and pasteurization can affect polyphenol contents of commercial juices. According to Ritter *et al* (1992) and Karadeniz and Eksi (2001) reports, clarification also decreased the polyphenolic contents of commercial fruit juices. Polyphenol contents decreased constantly with the progress of the ripening, while in red coloured varieties it increased during the last ripening stage due to the maximal accumulation of anthocyanidines and flavonols (Marinova *et al* 2005).

The decrease of limonin from 6.9±0.1 to 3.52±0.1 ppm might be due to production of CO_2 during storage. Carbon dioxide at pressures of 21 to 41 MPA at 30 °C-60 °C for 1h resulted in an average removal of 25% of the limonin from navel orange juice (Kimball 1987). The limonin content increased from 6.9±0.1 to 6.97±0.2 ppm at room temperature. A gradual increase in limonin content in juice blends with storage period might be due to conversion of a chemical compound limonate-a-ring lactone (non-bitter) in to limonin (bitter) in the juice (Premi *et al* 1994). The decrease of naringin was 443±10 to 143.7±4 ppm at refrigerated temperature and 443±10 to 428.9±5 at room temperature, due to hydrolysis of naringin into rhamnose and prunin by α-L-rhamnosidase activity of yeast.

The alcohol production started after 10 days and gradually increased from 0.11±0.01% to 0.89±0.05% after 90 days. The CO_2 pressure 0.65±0.05 bar started building after 10 days and reached up to 1.46±0.06 bar after 90 days. Sensitivity of yeast cells to ethanol marginally increased on decreasing the pH from 6.0-3.0. During fermentation process, CO_2, alcohol and glycerol produced is proportional to the amount of sugar fermented. The yeast strain produced large amount of glycerol at the expense of ethanol represent an advantageous alternative for the development of beverages with low ethanol content versus physical process which alter the organoleptic properties of the final product (Jairath *et al* 2012). Total yeast count was increased from $58x10^5$±10 to $58x10^8$±20 cfu mL^{-1} at the end of 90 days at refrigerated temperature. Total yeast count was not observed in beverge stored at room temperature because pasteurization kills the yeast cells. This study indicated that the shelf life of beverage was 90 days.

Table 4.16: Effect of storage time on microbiological and physicochemical properties of kinnow beverage (stored at refrigerated temperature)

Parameters	Days									
	Fresh	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
TSS (ºB)	13±0.2	12.8±0.1	12.5±0.2	12.4±0.3	12.2±0.3	12±0.3	11.8±0.3	11.5±0.2	11.4±0.2	11.2±0.3
Acidity (%)	0.14±0.03	0.21±0.02	0.32±0.04	0.36±0.02	0.4±0.04	0.45±0.03	0.49±0.02	0.51±0.03	0.56±0.01	0.58±0.01
pH	3.4±0.1	3.3±0.2	3.3±0.1	3.2±0.2	3.2±0.2	3.2±0.1	3.1±0.1	3.1±0.1	3.1±0.1	3.0±0.1
Brix-acid ratio	92.85±0.0	60.95±0.0	39.06±0.0	34.44±0.0	30.5±0.0	26.66±0.0	24.08±0.0	22.54±0.0	20.35±0.0	19.31±0.0
Total sugars (%)	12.9±0.3	12.4±0.4	12.3±0.4	11.9±0.4	11.7±0.2	11.2±0.2	10.9±0.5	10.1±0.3	9.8±0.3	9.5±0.4
Reducing sugars (%)	2.42±0.2	2.26±0.1	2.1±0.2	1.58±0.4	1.56±0.1	1.54±0.5	1.49±0.1	1.37±0.1	1.35±0.2	1.32±0.1
Ascorbic acid (mg/100 mL)	27.8±1	26.4±1	16.8±0.2	14.5±0.5	10.5±0.5	8.5±0.5	7.72±0.2	7.54±0.1	6.78±0.2	6.48±0.4
Total polyphenol contents (mg GAE/100 mL)	53.48±3	51.7±1	50.1±2	48.7±3	47.9±1	45.79±4	44.5±2	43.9±3	42.1±1	40.8±2
Limonin (ppm)	6.9±0.1	6.2±0.2	5.7±0.2	5.4±0.2	5.1±0.1	4.7±0.2	4.48±0.2	4.15±0.1	3.8±0.1	3.52±0.1
Naringin (ppm)	443±10	420.5±5	376.4±4	284.5±6	213±7	178±6	160.6±8	155.4±5	148.9±2	143.7±4
Alcohol (%,v/v)	0±0.0	0.11±0.01	0.35±0.03	0.54±0.02	0.63±0.03	0.72±0.3	0.75±0.4	0.8±0.2	0.82±0.3	0.89±0.05
CO_2 (bar)	0±0.0	0.65±0.05	0.72±0.02	0.82±0.02	1.16±0.04	1.19±0.01	1.21±0.03	1.25±0.05	1.33±0.03	1.46±0.06
Total yeast count (cfu mL^{-1})	$58x10^5$±10	$64x10^5$±20	$49x10^6$±10	$52x10^6$±10	$65x10^6$±20	$23x10^7$±10	$33x10^7$±20	$46x10^7$±20	$52x10^8$±10	$58x10^8$±20

Mean values ± standard error of three independent experiments

Table 4.17: Effect of storage time on microbiological and physicochemical properties of kinnow beverage (stored at room temperature after pasteurization)

Parameters	Days									
	Fresh	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
TSS (ºB)	13±0.2	13.1±0.2	13.2±0.2	13.5±0.1	13.7±0.1	13.8±0.1	13.85±0.2	13.9±0.2	13.95±0.2	13.95±0.1
Acidity (%)	0.14±0.03	0.25±0.04	0.35±0.03	0.39±0.02	0.45±0.01	0.49±0.02	0.53±0.02	0.54±0.02	0.59±0.03	0.60±0.03
pH	3.4±0.1	3.4±0.1	3.2±0.2	3.2±0.3	3.2±0.2	3.2±0.2	3.1±0.2	3.1±0.1	3.1±0.1	3.1±0.3
Brix-acid ratio	92.85±0.0	52.4±0.0	37.71±0.0	34.61±0.0	30.44±0.0	28.16±0.0	26.13±0.0	25.74±0.0	23.64±0.0	23.25±0.0
Total sugars (%)	12.9±0.3	12.95±0.2	13.2±0.3	13.3±0.3	13.3±0.2	13.3±0.2	13.4±0.3	3.4±0.4	13.45±0.2	13.5±0.3
Reducing sugars (%)	2.42±0.2	2.48±0.3	2.55±0.1	2.57±0.1	2.59±0.4	2.59±0.4	2.62±0.5	2.64±0.2	2.67±0.1	2.69±0.2
Ascorbic acid (mg/100 mL)	27.8±1	26.1±0.5	14.2±0.2	12.1±0.4	9.7±0.4	8.2±0.3	7.4±0.2	7.1±0.4	6.2±0.1	5.9±0.5
Total polyphenol contents (mg GAE/100 mL)	53.48±3	50.1±3	49.2±3	47.9±4	45.4±4	42.29±3	40.1±1	39.6±4	37.5±5	35.4±3
Limonin (ppm)	6.9±0.1	6.9±0.2	6.9±0.4	6.91±0.4	6.92±0.3	6.94±0.3	6.95±0.2	6.95±0.2	6.95±0.3	6.97±0.2
Naringin (ppm)	443±10	427±3	428.2±5	428.5±5	428.6±6	428.7±7	428.7±6	428.8±4	428.8±6	428.9±5
Alcohol (%,v/v)	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
CO_2 (bar)	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
Total yeast count (cfu mL^{-1})	$58x10^5$±10	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0

Mean values ± standard error of three independent experiments

4.12.3.1.1.1 Comparison of physicochemical properties of kinnow juice, debittered kinnow juice and debittered kinnow beverage stored at room temperature

Kinnow juice

- Juice with bitter component (naringin) above the permissible level (600 ppm).
- Short shelf life (two days) than debittered kinnow juice and beverage.
- No carbonation.
- Absence of rhamnose, prunin and naringenin as bioactive compounds.
- Less dietary vitamins and nutritive components than debittered kinnow juice and beverage **(Table 4.18)**.

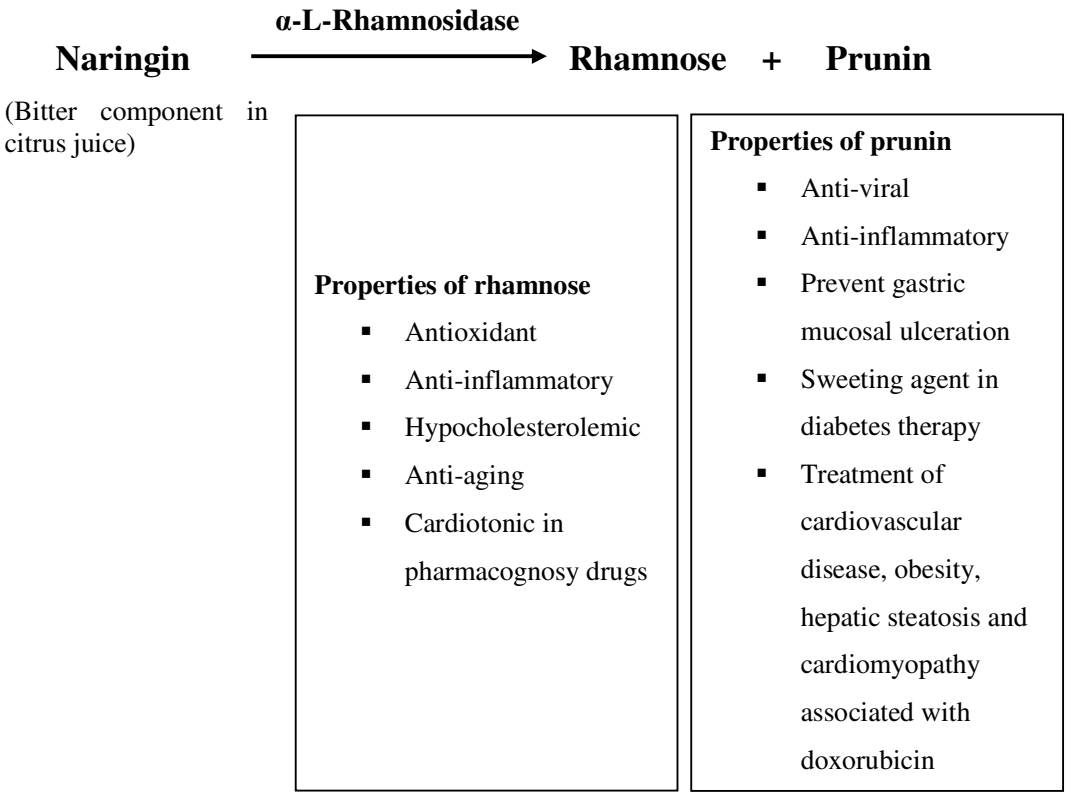
Debittered kinnow juice (juice + purified enzyme)

- Debittered, palatable, juice with bitter components below the permissible level (600 ppm) than kinnow beverage and juice.
- Long shelf life (30 days) than kinnow juice.
- A physiological benefit offers more variety of flavor and nutrients than kinnow beverage and juice.
- No preservative added.
- Minimally processed.
- New formulations as health drinks.
- No carbonation.
- Simple process parameters than kinnow beverage.
- Enriched with dietary vitamins and nutritive components of kinnow fruit
- Endowed with bioactive compounds rhamnose and prunin, formed due to hydrolysis of naringin by α-L-rhamnosidase activity **(Table 4.18)**.

Table 4.18: Comparison of physicochemical properties of kinnow juice, debittered kinnow juice (using purified enzyme) and kinnow beverage (using yeast) stored at room temperature

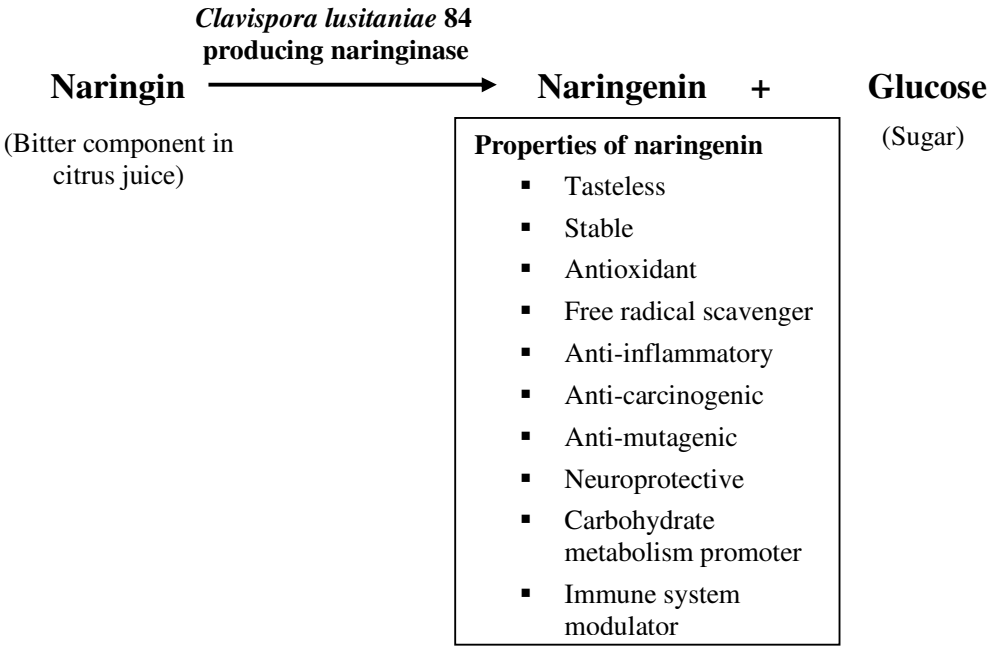
Parameters	Days					
	Fresh			10		
	Kinnow juice (control)	Kinnow juice+enzyme (0.8 IU mL^{-1})	Kinnow beverage (yeast inoculum-0.75% v/v)	Kinnow juice (control)	Kinnow juice+enzyme (0.8 IU mL^{-1})	Kinnow beverage (yeast inoculum-0.75% v/v)
TSS (ºB)	13±0.1	13±0.4	13±0.2	13.7±0.2	13.5±0.4	13.1±0.2
Acidity (%)	0.15±0.04	0.14±0.02	0.14±0.03	0.29±0.02	0.27±0.03	0.25±0.04
pH	3.5±0.4	3.5±0.3	3.4±0.1	3.4±0.3	3.4±0.1	3.4±0.1
Brix-acid ratio	86.66±0.0	92.85±0.0	92.85±0.0	47.24±0.0	50±0.0	52.4±0.0
Total sugars (%)	13.2±0.4	13.0±0.2	12.9±0.3	13.25±0.4	13.1±0.3	12.95±0.2
Reducing sugars (%)	2.46±0.4	2.45±0.1	2.42±0.2	2.58±0.5	2.53±0.2	2.48±0.3
Ascorbic acid (mg/100 mL)	26.4±3	27.7±2	27.8±1	24.2±0.4	25.5±0.4	26.1±0.5
Total polyphenol contents (mg GAE/100 mL)	51.12±2	52.23±4	53.48±3	46.7±3	48.58±1	50.1±3
Limonin (ppm)	7.1±0.2	6.7±0.2	6.9±0.1	7.4±0.4	6.98±0.3	6.9±0.2
Naringin (ppm)	467±8	397±6	443±10	567.4±4	248.5±5	427±3

Mean values ± standard error of three independent experiments



Kinnow beverage (Juice + yeast)

- Debittered, palatable, beverage with bitter components below the permissible level than kinnow juice.
- Long shelf life (90 days) than kinnow juice.
- A physiological benefit offers more variety of flavor and nutrients than kinnow juice.
- Replacement of synthetic colas with nutritional enriched carbonated beverages.
- No preservative added.
- Minimally processed.
- New formulations as health drinks.
- Sparkling, tangy taste, effervescence and antimicrobial due to carbonation.
- Simple process parameters and short duration fermentation.
- Value addition of distorted not fit for table purpose, small sized, immature dropped fruit.
- Enriched with dietary vitamins and minerals of fermenting microrganisms and nutritive components of kinnow fruit.
- Endowed with bioactive compounds naringenin and glucose formed due to hydrolysis of naringin by yeast **(Table 4.18)**.



4.12.3.1.2 Evaluation of sensory attributes of beverage during storage

The changes in sensory attributes like taste, color, aroma, bouquet, flavor and astringency of kinnow beverage were analyzed once every 10 days. All the sensory parameters were stable at storage period (90 days) with almost no change in organoleptic sensation **(Table 4.19 and Table 4.20)**. The beverage had pleasing effervescent sparkling orange colored appearance. Beverage was found to be acceptable up to 3 months of storage. The storage temperature can greatly affect the beverage tastes and smells. Lower temperatures will emphasize acidity and tannins while muting the aromatics. Higher temperatures will minimize acidity and tannins while increasing the aromatics. The presence of yeast in beverage gave a desirable freshness to the fermented beverage due to production of carbon dioxide and ethanol. Bhardwaj (2013) also reported that the low temperature and high relative humidity did not cause any change in qualitative characters and palatability of stored juice and helped in maintaining juice flavor, colour, TSS: acid ratio and sugars in balanced form than the ambient storage condition. In ambient condition change in colour of kinnow juice might be attributed to oxidation of phenolic compounds present in juice and maillard reaction between sugars and amino acids (Gonzalez 2000). A gradual decrese in flavour and taste which may be due to the degradation of ascorbic acid and furfural production and may also be due to heat treatment applied during processing (Pruthi *et al* 1983).

Table 4.19: Effect of storage time on organoleptic properties of kinnow beverage (at 4 °C)

Sensory attributes	Days									
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Appearance	7.4±0.1	7.4±0.2	7.3±0.1	7.3±0.2	7.4±0.2	7.3±0.2	7.3±0.1	7.4±0.2	7.3±0.2	7.3±0.1
Taste	7.9±0.4	8.1±0.3	8.2±0.2	8.1±0.2	8.1±0.3	7.8±0.2	7.8±0.1	7.8±0.4	7.9±0.5	7.9±0.4
Colour	8.0±0.2	8.0±0.1	8.0±0.3	8.0±0.2	7.9±0.4	7.8±0.1	7.8±0.1	7.9±0.1	7.8±0.2	7.8±0.2
Aroma	8.6±0.3	8.7±0.2	8.75±0.1	8.73±0.2	8.7±0.2	8.5±0.2	8.7±0.1	8.6±0.2	8.4±0.2	8.4±0.1
Bouquet	7.24±0.2	7.25±0.1	7.35±0.2	7.38±0.1	7.3±0.2	7.36±0.2	7.38±0.1	7.35±0.2	7.28±0.1	7.28±0.2
Body	7.4±0.2	7.45±0.3	7.45±0.2	7.48±0.3	7.42±0.2	7.45±0.3	7.45±0.1	7.42±0.1	7.4±0.2	7.48±0.1
Flavor	8.4±0.3	8.5±0.1	8.55±0.2	8.6±0.2	8.6±0.3	8.0±0.2	8.0±0.1	7.5±0.1	7.4±0.2	7.2±0.1
Astringency	7.5±0.1	7.6±0.2	8.0±0.3	8.4±0.1	8.5±0.2	8.5±0.3	8.5±0.1	8.2±0.2	8.3±0.2	8.2±0.2
Overall acceptability	8.2±0.1	8.3±0.2	8.4±0.1	8.4±0.2	8.2±0.1	8±0.3	7.9±0.3	7.8±0.2	7.8±0.1	7.8±0.05

*Mean value of three replicates

Like extremely 9, Like very much 8, Like moderately 7, Like slightly 6, Neither like/Dislike 5, Dislike slightly 4, Dislike moderately 3, Dislike very much 2, Dislike extremely 1

Plate 4.16 Extracted pectin from kinnow peel

Table 4.20: Effect of storage time on organoleptic properties of kinnow beverage (at room temperature)

Sensory attributes	Days									
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Appearance	7.4±0.1	7.2±0.1	7.2±0.1	7.2±0.1	7.1±0.1	7.1±0.1	7.1±0.2	7.1±0.2	7.0±0.1	7.0±0.1
Taste	7.9±0.4	7.9±0.3	7.8±0.1	7.8±0.1	7.8±0.2	7.7±0.2	7.7±0.3	7.6±0.3	7.6±0.4	7.3±0.3
Colour	8.0±0.2	7.9±0.2	7.9±0.4	7.9±0.4	7.7±0.3	7.7±0.3	7.7±0.2	7.5±0.2	7.5±0.2	7.1±0.2
Aroma	8.6±0.3	8.0±0.4	7.92±0.3	7.9±0.2	7.8±0.2	7.8±0.4	7.6±0.3	7.6±0.3	7.4±0.1	7.2±0.3
Bouquet	7.24±0.2	7.24±0.4	7.2±0.3	7.1±0.2	7.1±0.1	7.1±0.2	7.0±0.2	7.0±0.3	7.0±0.2	7.0±0.4
Body	7.4±0.2	7.4±0.1	7.4±0.1	7.4±0.1	7.35±0.3	7.35±0.3	7.35±0.2	7.3±0.2	7.3±0.3	7.1±0.3
Flavor	8.4±0.3	7.95±0.2	7.85±0.3	7.8±0.3	7.8±0.3	7.7±0.1	7.5±0.2	7.4±0.1	7.2±0.2	7.0±0.2
Astringency	7.5±0.1	7.4±0.1	7.4±0.2	7.4±0.1	7.3±0.3	7.3±0.1	7.2±0.2	7.2±0.3	7.0±0.1	7.0±0.1
Overall acceptability	8.2±0.1	7.9±0.3	7.8±0.4	7.8±0.2	7.75±0.2	7.75±0.4	7.7±0.2	7.7±0.3	7.5±0.2	7.5±0.02

*Mean value of three replicates

Like extremely 9, Like very much 8, Like moderately 7, Like slightly 6, Neither like/Dislike 5, Dislike slightly 4,

Dislike moderately 3, Dislike very much 2, Dislike extremely 1

4.12.3.2 Mineral Analysis of kinnow beverage

The result of mineral analysis of kinnow beverage before and after fermentation is presented in **Table 4.21**. A fermented debittered kinnow beverage contributes dietary minerals e.g. K, Ca, Mg, Fe, Zn, Cu and P. These metal ions are essential for regulating and building the living cells and aids in fighting depression thus important for their role in human nutrition. Magnesium helps in keeping the muscles relaxed, required for cell metabolism and helps to control the blood pressure and nerve transmitter. Calcium is required for normal contraction of muscles, heart, nervous activity and blood clotting. Microelements like Zn, Cu and Mn play an important role in the cellular metabolism. Zn act as cofactor of enzymes and Cu plays an important role in iron absorption and is involved in crosslinking of connective tissues, neurotransmission and lipid metabolism.

Metal ions govern the rate of sugar conversion to ethanol, the degree of attenuation, final ethanol yield, the amount of yeast biomass produced, cell viability and stress tolerance. Important metals that influence yeast fermentation are K and Mg (macroelements), Ca, Mn, Fe, Cu and Zn (microelements) which have marked effect on stability and dynamics of cell membrane, leading to downstream effect on cell permeability and signaling system. Yeast are known for their ability to accumulate metal ions from fruit beverage by different physicochemical interactions e.g. by adsorption and absorption, chelation, binding or by metabolism dependent mechanism (Walker *et al*. 1990). Sorption process is dependent on disposable functional groups on the cell surface and on the nature of metal ions. Therefore growth in different fruit beverage influences the capacity and selectivity of metal uptake by creating other binding sites or diverse enzymatic systems within the cells. Yeast effectively accumulate essential minerals and exclude or detoxify non-essential minerals.

Table 4.21: Mineral analysis of kinnow beverage

Minerals	**Kinnow beverage before fermentation (mg Kg^{-1})**	**Kinnow beverage after fermentation (mg Kg^{-1})**
Potasium	713.17	675.4
Calcium	71.56	70.25
Magnesium	66.13	60.92
Sodium	22.18	19.89
Iron	-	0.73
Zinc	0.69	-
Manganese	0.48	-

4.12.4 Pectin extraction

Pectin was isolated from the dried kinnow peel powder (25 g). Percentage yield was found to be around 0.82%. It was then stored in desiccators until further use. Similarly, Khule *et al* (2012) extracted pectin from citrus fruit peel and used as natural binder in paracetamol tablet **(Plate 4.16)**.

Chapter - V

SUMMARY

Enzyme α-L-rhamnosidase holds potential for debittering of citrus juice due to its ability to hydrolyze bitter naringin to non-bitter prunin and rhamnose which also enhances the aroma of citrus juice. The present study has been carried out to optimize parameters for complete bioprocess development for fermentative production of debittered kinnow juice and beverage using α-L-rhamnosidase producing yeast in order to utilize its immense potentiality in processed kinnow juice industry.

A total of thirty yeast strains were isolated from the whey beverage and screened for α-L-rhamnosidase enzyme production. Only four isolates (84, B82, 86 and S82) produced α-L-rhamnosidase enzyme by hydrolyzing naringin. Isolate 84 exhibited (0.058±0.02 IU mL^{-1}) maximum activity and S 82 exhibited (0.029±0.00 IU mL^{-1}) minimum activity.

All potential yeast isolates 84, B82, 86 and S82 have been identified by phenotypic and molecular characterization (18S rRNA gene partial sequencing) as *Clavispora lusitaniae* (KF633446), *Clavispora lusitaniae* (KF633447), *Candida* sp. YS12A (KF680225) and *Candida hyderabadensis* (K680226). Intra-species genetic diversity among isolates have been evaluated by whole genome analysis, using repetitive DNA sequences (ERIC, REP and BOX) based DNA fingerprinting, maximum numbers of amplified bands are shown by *Clavispora lusitaniae* (14, 7, 8) followed by *Clavispora lusitaniae* (11, 7, 5), *Candida hyderabadensis* (10, 7, 6) and *Candida sp*. YS12A (8, 5, 4), respectively.

All potential yeast strains could ferment D-glucose, D-galactose, D-raffinose, D-xylose and sucrose. Scanning electron microscopy revealed different morphotypes of *Clavispora lusitaniae* in presence of inducer naringin (0.2 %).

The sole carbon source utilization pattern of potential yeast strains (*Clavispora lusitaniae, Clavispora lusitaniae, Candida* sp. YS12A and *Candida hyderabadensis*) revealed 44, 42, 39 and 37 carbon sources utilization, respectively.

Clavispora lusitaniae produced 0.106 IU mL^{-1} enzyme activity in optimized minimal media containing rhamnose (0.6 % w/v), yeast extract (0.4% w/v), temperature (35 °C) and pH (4) after 48 h.

The yeast inoculum concentration (0.75% v/v), degree brix (13 °B), temperature (30 ±5 °C) and incubation time (48 h) depicted maximum α-L- rhamnosidase enzyme production in kinnow juice.

The bioprocess for purified α-L- rhamnosidase enzyme production includes production (0.106 IU mL^{-1}), 30-60% salt precipitation (1.30 IU mL^{-1}), dialysis (6.67 IU mL^{-1}) ion exchange chromatography (4.98 IU mL^{-1}) and lyophilization (8.2 IU mL^{-1}) a promising means to prepare the purified α-L-rhamnosidase commercially and setting up a strong base to

enzymatically debitter citrus juice. The enzyme has been purified to 10.1-fold with 46.98% recovery, having 24.9 IU activity and 2.7 IU mg^{-1} specific activity. Molecular weight of purified enzyme is 85 kDa by SDS PAGE. The pH and temperature optima of enzyme are 4 and 50 °C. The α-L rhamnosidase activity is strongly inhibited by Ag^{+} and slightly inhibited by Mg^{++}, Cu^{++} and Zn^{++} cations. Other cations Mn^{++}, Ca^{++}, Co^{++}, K^{+} and Na^{+} slightly activated the enzyme activity. The Km and Vmax values of purified enzyme are 0.18 mM and 25 IU mL^{-1} using p-nitrophenyl α-L-rhamnopyranoside as the substrate from the double reciprocal plots.

The purified enzyme has been found to be nontoxic and safe for food use. The result of metal analysis of purified enzyme shows that heavy metals As, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in the enzyme powder are 0.003 mg Kg^{-1}, 0.001 mg Kg^{-1}, 0.105 mg Kg^{-1}, 0.285 mg Kg^{-1}, 3.162 mg Kg^{-1}, 0.109 mg Kg^{-1}, 0.067 mg Kg^{-1}, 0.011 mg Kg^{-1} and 0.391 mg Kg^{-1}, the concentration is below permitted maximum levels for food additives.

The enzyme (0.8 IU mL^{-1}) in citrus juice could hydrolyze 600 ppm of naringin to palatable bitterness (180.71 ppm). The optimized parameters for debittering of kinnow juice are; enzyme activity (0.8IU mL^{-1}), TSS (13 °B), temperature (30±5°C) and incubation time (4 h) and for low alcoholic naturally carbonated beverage (LANC); yeast inoculum concentration (0.75 % v/v), TSS (13 °B), temperature (30±5 °C) and incubation time (48 h) with enhanced shelf life. The parameters of low alcoholic naturally carbonated beverage (LANC) after 90 days are; juice concentration (40%), TSS (11.2 °B), brix acid ratio (19.31), pH (3.0), acidity (0.56%), total sugars (9.5%), reducing sugars (1.32%), ascorbic acid (6.48 mg/100 mL), limonin (3.52 ppm), naringin (143.7 ppm), polyphenol contents (40.8 mg GAE/100 mL), alcohol (0.89 %), CO_2 pressure (1.46 bar) and total yeast count ($58x10^{8}$ cfu mL^{-1}).

On the basis of these results, it is concluded that bio-enzymatic debittering method by *Clavispora lusitaniae* can become the main direction of citrus juice debittering in the future, due to its characteristics of high specificity, preferable retention of juice flavor, nutrients and economical viable with strong ability to remove the bitter taste from citrus juice.

REFERENCES

Abbatea E, Palmeri R, Todaroa A, Blancoc R M and Spagnaa G (2012) Production of an α-L-rhamnosidase from *Aspergillus terreus* using citrus solid waste as inducer for application in juice industry. *Che Eng Transac* **27**:253-58.

Abdullakasim P S, Songchitsomboon M, Techagumpuch N, Balee P, Swatsitang and Sungpuag P (2007) Antioxidant capacity, total phenolics and sugar contents of selected Thai health beverages. *Int J Food Sci Nutr* **58**:77-85.

Ahmed M, Ahmad A, Chatha Z A and Dilshad S M R (2008) Studies on preparation of ready to serve mandarin (*Citrus reticulata*) diet drink. *Pak J Agri Sci* **45**:470-76.

Akhtar S, Riaz M, Ahmad A and Nisar (2010) A physico-chemical, microbiological and sensory stability of chemically preserved mango pulp. *Pak J Bot* **42**:853-62.

Amerine M A, Pangborn R M and Roessler E B (1965) *Principles of sensory evaluation of food.* Pp 602. Academic Press, Elsevier, London, UK.

Anonymous (2010) Available at www.marketpublisher.com.

AOAC (16th ed) (1999) *Official methods of analysis of the association of official analytical chemists*, AOAC International, Gaithersburg.

AOVC (1996) *Methods of vitamin assay, Association of vitamin chemists inc*. Pp 306-12. Interscience Publishers, New York.

Avila M, Jaquet M, Moine D, Requena T, Pelaez C and Arigoni F (2009) Physiological and biochemical characterization of the two α-L-rhamnosidases of *Lactobacillus plantarum* NCC245. *Microbiol* **155**:2739-49.

Banerjee G D (2009) Poised for a golden revolution. *Times Agriculture Journal*, 01 April. *Critical Reviews in Biotechnology* **16**:145-55 (1996).

Barnett J A, Payne R W and Yarrow D (2000) *Yeasts: characteristics and identification*. Cambridge University Press, Cambridge.

Bar-Peled M, Fluhr R and Gressel J (1993) Juvenile-specific localization and accumulation of a rhamnosyltransferase and its bitter flavonoid in foliage, flowers, and young citrus fruits. *Plant Physiol* **103**:1377-84.

Bauer S, Vasu P, Persson S, Mort A J and Somerville C R (2006) Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc Natl Acad Sci USA* **103**:11417-22.

Beekwilder J Marcozzi D and Vecchi S (2009) Characterization of rhamnosidases from *Lactobacillus plantarum* and *Lactobacillus acidophils*. *Appl Environ Microbiol* **75**:3447-54.

Berhow M A, Tisserat B, Kanes K and Vandercook C (1998) *Survey of Phenolic Compounds produced in Citrus*: Tech Bull. US Department of Agriculture/Agricultural Research Service, 1856, Washington DC: US Government Printing Office.

Bhardwaj R L (2013) Physico-chemical, sensory and microbiological quality of kinnow juice stored in refrigerated storage condition. *Asian J Dairy Food Res* **32**:203-13.

Bhardwaj R L and Mukherjee S (2011) Effects of fruit juice blending ratios on kinnow juice preservation at ambient storage condition. *Afr J Food Sci* **5**:281-86.

Birgisson H, Hreggvidsson G O, Fridjonsson O H, Mort A, Kristjansson J K and Mattiasson B (2004) Two new thermostable α-L-rhamnosidases from a novel thermophilic bacterium. *Enzyme Microb Technol* **34**:561-71.

Bisswanger H (3rd ed) (1999). *Enzymkinetik: Theorie und methoden Weinheim: Wiley-VCH.*

Bourbouze R, Perchekon F and Courtois J E (1976) α-L-Rhamnosidase from *Fagopyrum esculentum*: purification and some properties. *Eur J Biochem* **63**:331-37.

Bourbouze R, Pratviel-Sosa F and Percheron F (1975) Rhamnodiastase et α-L-rhamnosidase de *Fagopyrum esculentum*. *Phytochem* **14**:1279-82.

Bouriche H and Arnhold J (2010) Effect of Cleome arabica leaf extract treated by naringinase on human neutrophil chemotaxis. *Nat Prod Commun* **5**:415-18.

Bram B and Solomons G L (1965) Production of the enzyme naringinase by *Aspergillus niger*. *Appl Environ Microbiol* **13**:842-45.

Busto M D, Meza V and Mateos N P (2007) Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly (vinyl alcohol) cryogels for the debittering of juices. *Food Chem* **104**:1177-82.

Caputi A J, Veda M and Brown T (1968) Spectrophotometric determination of ethanol in wine. *Am J Enol Vitic* **19**:160-65.

Castillo J, Benavente-Garcia O and Del Rio J A (1992) Naringin and neohesperidin levels during development of leaves, flower buds, and fruits of *Citrus aurantium*. *Plant Physiol* **99**:67-73.

Chandler B V and Kefford J F (1968) Removal of limonin from bitter orange juice. *J Sci Food Agric* **19**:83.

Chandler B V and Nicol K J (1975) Some relationships of naringin: their importance in orange juice bitterness. *CSIRO Food Res Quart* **35**:79-88.

Chaplin M F and Bucke C (1990) *Enzyme Technology*. Cambridge University Press, Cambridge. This book is out of print, an electronic version is updated by M. Chaplin and available at: http://www.sbu.ac.uk/biology/enztech.

Chen D, Niu T and Cai H (2010) Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316. *Afr J Biotechnol* **9**:4970-78.

Chien P J, Sheu F and Shiyu Y T (2001) Monitoring enzymatic debittering in grapefruit juice by high performance liquid chromatography. *J Food and Drug Anal* **9**:115-20.

Cornish-Bowden A (1976) Basic principles of chemical kinetics. Pp 145-155. In: Cornish-Bowden A (ed) *Principles of Enzyme Kinetics*. Butterworth & Co, London (Original not seen.1996. *Critical Reviews in Biotechnology* **16**:7-9.

Cubero O F, Crespo A, Fatehi J and Bridge P D (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium-store lichenized and other fungi. *Plan Sys Evol* **216**:243-49.

Cui Z, Maruyama Y, Mikami B, Hashimoto W and Murata K (2007) Crystal structure of glycoside hydrolase family 78 α-Lrhamnosidase from *Bacillus* sp. GL1. *J Mol Biol* **374**:384-98.

Davis D W 1947 Determination of flavonones in citrus juice. *Anal Chem* **19**:46-48.

Del Rio J A, Gomez P, Baidez A G, Arcas M C, Botia J M and Ortuno A (2004) Changes in the levels of polymethoxyflavones and flavanones as part of the defense mechanism of Citrus sinensis (Cv. Valencia Late) fruits against *Phytophthora citrophthora*. *J Agric Food Chem* **52**:1913-17

Dilipkumar K S and Yashi S (2014) Preparation and quality characteristics of carbonated beverage prepared by profiteered, ultra filtered acidic whey with different fruit juices. *International J of innovative research in technology&science* **2**:52-60.

Dubois M, Giles K A, Hamilton J K, Rebers P A and Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**:350-56.

Eijsink V, Gaseidnes S B and Van den Burg B (2005) Directed evolution of enzyme stability. *Biomol Eng* **22**:21-30.

Elinbaum S, Ferreyra H, Ellenrieder G and Cuevas C (2002) Production of *Aspergillus terreus* α-L-rhamnosidase by solid state fermentation. *Lett Applied Microb* **34**:67-71.

FAO/WHO (2011) Joint FAO/WHO food standards programme codex committee on contaminants in foods. FAO/WHO, Netherlands.

Fayoux S C, Hernandez R J and Holland R V (2007) The debittering of navel orange juice using polymeric films. *J Food Sci* **72**:143-54.

Feng B, Hu W, Ma B, Wang Y, Huang H and Wang S (2007) Purification, characterization, and substrate specificity of a glucoamylase with steroidal saponin-rhamnosidase activity from *Curvularia lunata*. *Appl Microbiol Biotechnol* **76**:1329-38.

Feng B, Ma B, Kang L, Xiong C and Wang S (2005) The microbiological transformation of steroidal saponins by *Curvularia lunata*. *Tetrahedron* **61**:11758-63.

Ferreira L, Afonso C, Vila-Real H, Alfaia A and Ribeiro M H L (2008) Debittering of grapefruit juice with naringinase. *Food Technol Biotechnol* **46**:144-48.

Fukumoto J and Okada S (1973) Naringinase production by fermentation. Japanese Patent, **(306)**:554.

Gallego M V, Pinaaga F, Ramoan D and Valleas S (1996) Production and characterization of an *Aspergillus terreus* α-L-rhamnosidase of oenological interest. *Z Lebensm Unters Forsch* **203**:522-27.

Gallego M V, Pinaaga F, Ramoan D and Valleas S (2000) Purification and characterization of an α-L-rhamnosidase from *Aspergillus terreus* of interest in winemaking. *Journal of Food Science In press.*

Gallego M V, Pinaga F, Ramon D and Valles S (2001) Production and characterization of an *Aspergillus terreus* α-L-rhamnosidase of oenological interest. *J Food Sci* **66**:204-09.

Garnier P, Wang X T, Robinson M A, Perkins A C, Frier M and Davis B G (2010) Lectin directed enzyme activated prodrug therapy: synthesis and evaluation of rhamnose capped prodrugs. *J Drug Targeting* **18**:794-02.

Gattuso G, Barreca D, Gargiulli C, Leuzzi U and Caristi C (2007) Flavonoid composition of Citrus juices. *Molecules* **12**:1641-73.

Ghosh S K (2011) Study of yeast flora from fruit of *Syzygium cumini* (linn) skeel. *Agric Biol J N Am* **2**:1166-70.

Giavasis I, Harvey L M and McNeil B (2000) Gellan gums. *Crit Rev Biotech* **20**:177-211.

Gonzalez E R and Leeson S (2000) An investigation on the preservation of Kunun-zaki, an African fermented cereal based food drink. *Acta Alimentaria* **29**:385-92.

Griffith F P (1969) Process for Reactivating Polyamides Resin Used in Debittering Citrus Juice. U.S. Patent, 3463763.

Guadagni D G, Maier V P and Turnbaugh J G (1973) Effect of some citrus juice constituent on taste thresholds for limonin and naringinin bitterness. *J Sci Food Agric* **24**:1277-88.

Hall D H (1938) A new enzyme of the glycosidase type. *Chem Ind* **57**:473.

Hall J A (1925) Glucosides of the navel orange. *J Am Chem Soc* **47**:1191-95.

Han X, Ren D, Fan P, Shen T and Lou H (2008) Protective effects of narigenin-7-O-glucoside on doxorubicin induced apoptosis in H9C2 cells. *Eur J Pharmacol* **581**:47-53.

Harborne J B (1967) Comparative biochemistry of the flavonoids. Academic Press Inc, London.

Hasegawa S, Suhayda C G, Hsu W J and Robertson G H (1997). *Phytochemistry* **46**:33-37.

Hashimoto W and Murata K (1998) α-L-Rhamnosidase of *Sphingomonas* sp. R1 producing an unusual exopolysaccharide of Sphingan. *Biosci Biotechnol Biochem* **62**:1068-74.

Hashimoto W, Miyake O, Nankai H and Murata K (2003) Molecular identification of an α-L-rhamnosidase from *Bacillus* sp strain GL1 as an enzyme involved in complete metabolism of gellan. *Arch Biochem Biophys* **415**:235-44.

Hashimoto W, Nankai H, Sato N, Kawai S and Murata K (1999) Characterization of α-L-rhamnosidase of *Bacillus* sp. GL1 responsible for the complete depolymerization of gellan. *Arch Biochem Biophys* **368**:56-60.

Hashinaga F, Ejima H, Nagahama H and Itoo S (1977) *Bull Fac Agric Kagoshima Univ* **27**:171-79.

Hierro N, Gonzalez A, Mas A and Guillamon J M (2004) New PCR-based methods for yeast identification. *J Appl Microbiol* **97**:792-01.

Horowitz R M and Gentili B (1961) Phenolic glycosides of grapefruit: a relation between bitterness and structure. *Arch Biochem Biophys* **92**:191-92.

Hughes H B, Morrissey J P and Osbourn A E (2004) Characterization of the saponin hydrolysing enzyme avenacosid α-L-rhamnosidase from the fungal pathogen of cereals, *Stagonospora avenae*. *Eur J Plant Pathol* **110**:421-27.

Hwang H J, Kim S W, Xu C P, Choi J W and Yun J W (2003) Production and molecular characteristics of four groups of exopolysaccharides from submerged culture of *Phellinus gilvus*. *J Appl Microbiol* **94**:708-19.

Islam M T, Hashidoko Y, Deora A, Ito T and Tahara S (2005) Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter sp.* strain SB-K88 is linked to plant colonization and antibiosis against soilborne Peronosporomycetes. *Appl Environ Microbiol* **71**:3786-96.

Jafari-Aghdam J, Khajeh K, Ranjbar B and Nemat-Gorgani M (2005) Deglycosylation of glucoamylase from *Aspergillus niger*: effects on structure, activity and stability. *Biochim Biophys Acta* **1750**:61-68.

Jagjiwan D (2001) *Physico-chemical debittering and processing of kinnow juice with by-product recovery*. M.Sc. thesis, Thapar Institute of Engineering and Technology, Patiala, India.

Jairath and Sahota P P (2011) Technology development for production of Non Alcoholic Naturally Carbonated beverage from fruit juices (PAT/FA/110019/2011, NRDC).

Jairath S, Sahota P P and Pandove G (2012) Preparation of non-alcoholic naturally carbonated beverage using yeast isolate from whey beverage. *Czech J Food Sci* **30**:135-43.

Jang I S and Kim D H (1996) Purification and characterization of α-L-rhamnosidase from *Bacteroidesn* JY-6, a human intestinal bacterium. *Biol Pharm Bull* **19**:1546-49.

Jawanda J S and Singh K K (1973) Kinnow holds out promise for Punjab. *Punjab Hort J* **13**:89-93.

Johnson R L and Chandler B V (1988) Absorptive removal of bitter principles and titrable acid from citrus juices. *Food Technol* **45**:130.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2004) Safety evaluation of certain food additives and contaminants. WHO Food Additives Series No 52.

Jooyandeh H (2013) Production of non-alcoholic fermented plum beverages. *M E J S R* **13**:836-43.

Jourdan P S, McIntosh C A and Mansell R L (1985) Naringin levels in citrus tissues: II. Quantitative distribution of naringin in *C. paradisi*. *Plant Physiol* **77**:903-08.

Jukes T H and Cantor C R (1969) Evolution of Protein Molecules. In: Munro H N (ed) *Mammalian Protein Metabolism*. pp 21-132. Academic Press, New York.

Kaji A and Ichimi T (1973) Alpha-L-Rhamnosidase activity in culture filtrate of *Corticium rolfsii*. Enzymatic activity at low pH. *Agric Biol Chem* **37**:431-32.

Kamaljeet K (2002) *Application of novel juice extraction methods and bacterial utilization of limonin for control of bitterness in kinnow juice*. M.Sc. Thesis, Thapar Institute of Engineering and Technology, Patiala, India.

Kaminarides S E and Laskos N S (1992) Yeasts in factory brine of feta cheese. *Aust J Dairy Technol* **47**:68-71.

Karadeniz and Eksi A (2001) Elma suyunda fenolik bilesiklerin proses ve depolama sirasinda deg-isimi, Gida **26**:255-60.

Karasu Yalcin S, Senses Ergul S and Yesim Ozbas Z (2012) Identification and enzymatic characterization of the yeasts isolated from Erzincan tulum cheese. *MLJEKARSTVO* **62**:53-61.

Kaul T, Middleton E and Ogra P L (1985) Antiviral effects of flavonoids on human viruses. *J Med Virol* **15**:71-79.

Kaur A, Singh S, Singh R S, Schwarz W H and Puri M (2010) Hydrolysis of citrus peel naringin by recombinant α-L-rhamnosidase from *Clostridium stercorarium*. *J Chem Technol Biotechnol* **85**:1419-22.

Kaur K (2002) *Application of novel juice extraction methods and bacterial utilization of limonin for control of bitterness in kinnow juice*. M.Sc. Thesis, Thapar Institute of Engineering and Technology, Patiala, India.

Khandelwal P, Kumar V, Das N and Tyagi S M (2006) Development of a process for preparation of pure and blended kinnow wine without debittering kinnow mandarin juice. *Internet J Food Safety* **8**:24-29.

Khule N R, Mahale N B, Shelar D S, Rokade M M and Chaudhari S R (2012) Extraction of pectin from citrus fruit peel and use as natural binder in paracetamol tablet. *Der Pharmacia Lettre* **4**:558-64.

Kim J H, Long M J C, Seo W D, Lee J H, Lee B W and Yoon Y J (2005) α-Rhamnosidase inhibitory activities of polyhydroxylated pyrrolidine. *Bioorg Med Chem Lett* **15**: 4282-85.

Kim S W, Hwang H J, Xu C P, Sung J M, Choi J W and Yun J W (2003) Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharide by *Cordyceps militaris* C738. *J Appl Microbiol* **94**:120-26.

Kimball D A and Norman S I (1990) Processing effects during commercial debittering of California navel juice. *J Agric Food Chem* **38**:1396.

Kimball D A and Seth N (1987) Changes in California naval orange juice during commercial debittering. *J Food Sci* **55**:273-74.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evolution* **16**:111-20.

Kita M, Hisada S, Endo‐Inagaki T, Omura M and Moriguchi T (2000) Changes in the levels of mRNAs for putative cell growth‐related genes in the albedo and flavedo during citrus fruit development. *The Plant Cell Reports* **19**:582-87.

Klibanov A M (1983) Stabilization of enzymes against thermal inactivation. *Adv Appl Microbiol* **29**:1-28.

Konno A, Misaki M, Toda J, Wada T and Yasumatsu K (1982) Bitterness reduction of naringin and limonin by β-cyclodextrin. *Agric Biol Chem* **46**:2203-08.

Koseki T, Mese Y, Nishibori N, Masaki K, Fujii T, Handa T, Yamane Y, Shiono Y, Murayama T and Lefuji H (2008) Characterization of an α-L-rhamnosidase from *Aspergillus kawachii* and its gene. *Appl Microbiol Biotechnol* **80**:1007-13.

Koseki T, Miwa Y, Mese Y, Miyanaga A, Fushinobu S, Wakagi T, Shoun H, Matsuzawa H and Hashizume K (2006) Mutational analysis of N-glycosylation recognition sites on the biochemical properties of *Aspergillus kawachii* α-L-arabinofuranosidase 54. *Biochim Biophys Acta* **1760**:1458-64.

Kreger-Van Rij N J W (1984) *The Yeast a Taxonomic Study*. Pp 1082. Elsevier Science Publishing Company, New York.

Kumar R, Kaushik R A and Chharia A S (1992) Effect of post harvest treatment on the quality of mango during storage. *Haryana J Hort Sci* **21**:49.

Kumar V (2010) Compartive studies on inducers in the production of naringinase from *Aspergillus niger* MTCC 1344. *Afr J Biotechnol* **9**:7683-86

Kurosawa Y, Ikeda K and Egami F (1973) Alpha-L-rhamnosidases of the liver of *Turbo cornutus* and *Aspergillus niger*. *J Biochem* **73**:31-37.

Kurtzman C P and Fell J W (1998) Definition, classification and nomenclature of the Yeasts. In: Kurtzman C P and Fell J W (ed) *The yeasts, a taxonomic study*. Pp 3. Elsevier, Amsterdam.

Kurtzman C P and Fell J W (4th ed) (1997) *The Yeasts, a taxonomic study*. pp 1055. Elsevier Science Publishing Company, Amsterdam.

Kurtzman C P and Fell J W (4th ed) (2000) *The yeasts a taxonomic study*. Elsevier Science, Amsterdam.

Kurtzman C P, Fell J W and Boekhout T (5th ed) (2011) *The Yeasts, A taxonomic study*. Vol 1. Elsevier Science, Amsterdam.

Kurtzman C P, Robnett C J, Ward J M, Bravton C, Gorelic P and Walsh T M (2005) Multigene phylogenetic analysis of pathogenic *Candida* species in the Kazachstania (Arxiozyma) telluris complex and description of their ascosporic states as *Kazachstania bovina* sp. nov., *K. heterogenica* sp. nov., *K. pintolopesii* sp. nov., and *K. slooffiae* sp. nov. *J Clin Microbiol* **43**:101-11.

Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-85.

Larkin M A, Blackshields G, Brown N P, Chenna R and Mc Gettigan P A (2007) Clustal W and Clustal X version 2. *Bioinform* **23**:2947-48.

Lei S, Xu Y, Fan G, Xiao M and Pan S (2011) Immobilization of naringinase on mesoporous molecular sieve MCM-41 and its application to debittering of white grapefruit. *Appl Surface Sci* **257**:4096-99.

Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951) Protein measurement with the olin phenol reagent. *J Biol Chem* **193**:265-75.

Magario I, Neumann A, Oliveros E and Syldatk C (2009) Deactivation kinetics and response surface analysis of the stability of α-L-rhamnosidase from *Penicillium decumbens*. *Appl Biochem Biotechnol* **152**:29-41.

Maier V P, Brewster L C and Hsu A C (1973) Ethylene accelerated limonoids metabolism in citrus fruits: a process for reducing juice bitterness. *Agric Food Chem* **21**:490.

Manlan M, Mathews R F, Rouseff R L, Littell R C, Marshall M R, Moye H A and Texeira A A (1990) Evaluation of the properties of polystyrene divinyl benzene adsorbents for debittering grapefruit juice. *J Food Sci* **55**:440.

Manzanares P, Broeck H C V, Graaff L H D and Visser J (2001) Purification and characterization of two different α-L-rhamnosidases, RhaA and RhaB from *Aspergillus aculeatus*. *Appl Environ Microbiol* **67**:2230-34.

Manzanares P, De Graaff L H, Visser J (1997) Purification and characterization of an α-L rhamnosidase from *Aspergillus niger*. *FEMS Microbial Lett* **157**:279-83.

Manzanares P, Orejas M, Gil J V, de Graaff L H, Visser J and Ramón D (2003) Construction of a genetically modified wine yeast strain expressing the *Aspergillus aculeatus* rhaA gene, encoding an α-L-rhamnosidase of enological interest. *Appl Environ Microbiol* **69**:7558-62.

Manzanares P, Orejas M, Ibanez E, Valless and Ramon D (2000) Purification and characterization of an α-L rhamnosidase from *Aspergillus nidulans*. *Lett Appl Microbiol* **31**:198-02.

Mapson L W (1970) Vitamins in fruits. In: Hulme A C (ed) *The Biochemistry of Fruits and their Products*. Pp 369-84. Academic Press, London.

Marinova D, Ribarova F and Atanassova M (2005) Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *J Univ Chem Technol Metallurgy* **40**:255-60.

Mayer V P and Beverly G D (1968) Limonin monolactone, the nonbitter precursor responsible for delayed bitterness in certain Citrus juices. *J Food Sci* **33**:488-92.

Mayer V P, Hasegawa S and Here E (1969) *Phytochemistry* **8**:405-07.

Mazzaferro L S, Orrillo G A Ledesma P and Breccia J D (2008) Dose-dependent significance of monosaccharides on intracellular *a*-L-rhamnosidase activity from *Pseudoalteromonas* sp. *Biotechnol Lett* **30**:2147-50.

McMahon H, Zoecklein B W, Fugelsang K and Jasinski Y (1999) Quantification of glycosidase activities in selected yeasts and lactic acid bacteria. *J Ind Microbiol Biotechnol* **23**:198-03.

Mehta U and Bajaj S (1983) Effect of storage and method of preservation on the physico-chemical characteristics of citrus juices. *Indian Food Packer* **37**:42-51.

Menon S S, Basavaraj B V, Bharath S, Deveswaran R, Madhavan V (2011) *Der Pharmacia Lettre* **3**:241-47.

Miake F, Satho T, Takesue H, Yanagida F, Kashige N and Watanabe K (2000) Purification and characterization of intracellular alpha-l-rhamnosidase from *Pseudomonas paucimobilis* FP2001. *Arch Microbiol* **173**:65-70.

Michlmayr H, Schümann C, Kulbe K D and del Hierro A M (2011) Heterologously expressed family 51 alpha-L-arabinofuranosidases from *Oenococcus oeni* and *Lactobacillus brevis*. *Appl Environ Microbiol* **77**:1528-31.

Miguel M G C P and Cardoso P G (2013) Identification and assessment of kefir yeast potential for sugar/ethanol-resistance. *Braz J Microbiol* **44**:113-18.

Miklosy E and Polos V (1995) Yeasts with β-D-glucosidase activity: properties and possible application in winemaking processes. *Acta Aliment* **24**:167-80.

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**:426-28.

Miyata T, Kashige N, Satho T, Yamaguchi T, Aso Y and Miake F (2005) Cloning, sequence analysis, and expression of the gene encoding *Sphingomonas paucimobilis* FP2001 α-L-rhamnosidase. *Curr Microbiol* **51**:105-09.

Mongkolkul P, Rodart P, Pipatthitikorn T, Meksut L and Sanguandeekul R (2006) Debittering of tangerine citrus *Reticulata Blanco* juice by *β*-cyclodextrin polymer. *J Incl Phenom Macrocycl Chem* **56**:167-70.

Monti D, Pisvejcova A, Kren V, Lama M and Riva S (2004) Generation of an α-L-rhamnosidase library and its application for the selective derhamnosylation of natural products. *Biotechnol Bioeng* **87**:763-71.

Montogomery DC (5th ed) (2000) *Design and Analysis of Experiments*. John Wiley and Sons, Singapore.

Mukund P, Prasanna D B and Saidutta M B (2014) Production of naringinase from a new soil isolate *Bacillus methylotrophicus*: isolation, optimization and scale-up studies. *Prep Biochem Biotechnol* **44**:146-63.

Mutter M, Beldman G, Schols H A and Voragen A G J (1994) Rhamnogalacturonan α-L-rhamnopyronohydrolase. A novel enzyme specific for the terminal nonreducing rhamnosyl unit in rhamnogalacturonan regions of pectin. *Plant Physiol* **106**:241-50.

Neter J, Kutner M H, Nachtsheim C J and Wasserman W (4th ed) (1996) *Applied Linear Statistical Models*. McGraw-Hill, New York.

NHB (2010) World Wide Web source for National Horticulture Board data.

NHM (2014) World Wide Web source for National Horticulture Mission data.

Ni H, Chen F, Cai H, Xiao A, You Q and Lu Y (2012) Characterization and preparation of *Aspergillus niger* naringinase for debittering citrus juice. *J Food Sci* **71**:1-7.

Norouzian D, Hosseinzadeh A, Inanlou D N and Moazami N (1999) Various techniques used to immobilize naringinase produced by *Penicillium decumbens* PTCC 5248. *World J Microb Biotechnol* **15**:501-02.

Norouzian D, Hosseinzadeh A, Nouri I D, Moazami N (2000) Production and partial purification of naringinase by *Penicillium decumbens* PTCC 5248. *World J Microb Biotechnol* **16**:471-73.

Orejas M, Ibanez E and Ramon D (1999) The filamentous fungus *Aspergillus nidulans* produce an α-L-rhamnosidase of potential oenological interest. *Lett. Appl Microbiol* **28**:383-88.

Orrillo A G, Ledesma P, Delgado O D, Spagna G and Breccia J D (2007) Cold-active α-L-rhamnosidase from psychrotolerant bacteria isolated from a sub-Antarctic ecosystem. *Enzyme Microb Technol* **40**:236-41.

Ortuno A, Garcia-Puig D, Fuster M D, Perez M L, Sabater F, Porras I, Garcia-Lidon, A and Del Rio J A (1995) Flavanone and nootkatone levels in different varieties of grapefruit and pummelo. *J Agri Food Chem* **43**:1-5.

Oyedeji K O, bolarinwa A F and Oladosu I A (2013) Effect of isolated tetracyclic steroid constituent of *Portulaca oleracea* on reproductive parameters in male rats. *Asian J Pharm Clin Res* **6**:225-29.

Ozaki Y, Ayano S, Inaba N, Miyake M, Berhow M A and Hasegawa S (1995) Limonoid glucosides in fruit, juice, and processing byproducts of Satsma mandarin (*Citrus unshiu* Marcov.). *J Food Sci* **60**:186-89.

Park S, Kim J and Kim D (2005) Purification and characterization of quercitrin-hydrolyzing alpha-l-rhamnosidase from *Fusobacterium* K-60, a human intestinal bacterium. *J Microbiol Biotechnol* **15**:519-24.

Patil M B and Dhake A B (2014) Debittering of citrus fruit juice by naringinase of *Penicillium purpurogenum. Int J Engg Res Sci Tech* **3**:266-70.

Peterson J J, Beecher G R, Bhagwat S A, Dwyer J T, Gebhardt S E, Haytowitz D B and Holden J M (2006a) Flavanones in grapefruit, lemons and limes: a compilation and review of the data from the analytical literature. *J Food Compost Anal* **19**:74-80.

Peterson J J, Dwyer J T, Beecher G R, Bhagwat S A, Gebhardt S E, Haytowitz D B and Holden J M (2006b) Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature. *J Food Compost Anal* **19**:66-73.

Petri A C, Buzato J B, Celligoi M A P C and Borsato D (2014) Optimization of the Production of α-LRhamnosidase by *Aspergillus niger* in solid state fermentation using agro-Industrial residues. *Br Microbiol Res J* **4**:1198-10.

Prakash S, Singhal R S and Kulkarni P R (2002) Enzymic debittering of Indian grapefruit (*Citrus paradis*) juice. *J Sci Food Agric* **82**:394-97.

Praphailong W, Gestel M V, Fleet G H and Heard G M (1997) Evaluation of the Biolog system for the identification of food and beverage yeasts. *Lett Appl Microbiol* **24**:455-59.

Premi B R, Lal B B and Joshi V K (1994) Distribution pattern of bittering principle in kinnow fruits. *J Food Sci Technol* **3**:140-41.

Puri A (1984) Preparation and Properties of Citrus Juices, Concentrates and Dried Powders Which Are Reduced in Bitterness. U.S. Patent, 4439458.

Puri J S, Kothari, R M and Kennedy J F (1996) Biochemical basis of bitterness in citrus fruits and biotech approaches for dibittering. *Critical Review in Biotech* **16**:145-55.

Puri M (2012) Updates on naringinase: structural and biotechnological aspects *Appl Microbiol Biotechnol* **93**:49-60.

Puri M and Banerjee U C (2000) Production, purification and characterization of the debittering enzyme naringinase. *Biotechnol Adv* **18**:207-17.

Puri M and Kalra S (2005) Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for transformation of flavoids. *World J Microbiol Biotechnol* **21**:753-58.

Puri M, Banerjee A and Banerjee U C (2005) Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344. *Process Biochem* **40**:195-01.

Puri M, Kaur A, Barrow C J and Singh R S (2011) Citrus peel influences the production of an extracellular naringinase by *Staphylococcus xylosus* MAK 2. *Appl Microbiol Biotechnol* **89**:715-22.

Puri M, Kaur A, Singh R S and Kanwar J R (2008) Immobilized enzymes for debittering citrus fruit juices. In: Busto M D and Ortega N (ed) *Food Enzymes: Application of New Technologies*. Pp 91-103. Transworld Research Network, Trivandrum, India.

Puri M, Kaur A, Singh R S and Nahar A (2010a) Response surface optimization for the production of naringinase from *Staphlococcus xylosus* MAK2. *Applied Biochem Biotechnol* **162**:181-91.

Puri M, Kaur A, Singh R S, Schwarz W H and Kaur A (2010b) One step purification and immobilization of His-tagged rhamnosidase for narigin hydrolysis. *Process Biochem* **45**:445-51.

Purthi J S, Mann J K and Teotia M S (1983). Studies on utilization of kinnow and malta oranges. *J Food Sci Technol* **21**:123-27.

Qian S, Yu H, Zhang C, Lu M, Wang H and Jin F (2005) Purification and characterization of dioscin-α-L-rhamnosidase from pig liver. *Chem Pharm Bull* **53**:911-14.

Rademaker J L W and de Bruijn F J (1997) Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In: Caetano-Anolles G and Gresshoff P M (ed) *DNA Markers: Protocols. Application and Overviews*. Pp 151-71. New York.

Rajal V B (2002) *Penicillium ulaiense. Estudios de crecimiento y de produccio´n de hidrolasas*. Ph.D. dissertation. Facultad de Ingenierı´a, Universidad Nacional de Salta, Argentina.

Rajal V B, Cid A G, Ellenrieder G and Cuevas C M (2009) Production, partial purification and characterization of α-L-rhamnosidase from *Penicillium ulaiense*. *World J Microbiol Biotechnol* **25**:1025-33.

Ranote P S and Bains G S (1982) Juice of kinnow fruit. *Indian Food Packer* **36**:23-33.

Rastogi N K, Nguyen L T, Jiang B and Balasubramaniam V M (2010) Improvement in texture of pressure-assisted thermally processed carrots by combined pretreatment using response surface methodology. *Food Bioprocess Tech* **3**:762-71.

Ribeiro M H (2011) Naringinases: occurrences, characteristics and applications. *Appl Microbiol Biotechnol* **90**:1883-95.

Ritter G, Maier G, Schöplein E and Dietrich H (1992) The application of polyphenol oxidase in processing of apple juice. *In Proceedings of the JIEP 92. XVI. Conference of the Group Polyphenols*. pp 1-4, Lisbao.

Robinson M A, Charlton S T, Garnier P, Wang X and Davis S S (2004) LEAPT: lectin directed enzyme-activated prodrug therapy. *Proc Nat Acad Sci* **40**:14527-32.

Roitner M, Schalkhammer T and Pittner F (1984a) Characterization of naringinase from *Aspergillus niger*. *Monatsh Chem* **115**:1255-67.

Roitner M, Schalkhammer T and Pittner F (1984b) Preparation of prunin with the help of inmobilized naringinase pretreated with alkaline buffer. *Appl Biochem Biotech* **9**:483-88.

Rojas N L, Voget C E, Hours R A, Cavalitto S F (2011) Purification and characterization of a novel alkaline α-L-rhamnosidase produced by *Acrostalagmus luteo albus*. *J Ind Microbiol Biotechnol* **38**:1515-22.

Romero C, Manjon A, Bastida J and Iborra J L (1985) A method for assaying rhamnosidase activity of naringinase. *Anal. Biochem* **149**:566-71.

Rosi I, Domizio P, Vinella M and Salicone M (1995) Hydrolysis of grape glycosides by enological β- glucosidases. In Charalambous G (ed) *Food flavors: Generation, Analysis and Process Influence*. Pp 1623-35. Eliesvier Science, Amsterdam.

Rousseff R L, Martin S F and Youtsey C O (1987) Quantitative survey of narirutin, naringin, hesperidin and neohesperidin in citrus. *J Agric Food Chem* **35**:1027-30.

Sahota P, Pandove G, Jairath S and Dhillon T S (2010a) Technology for the production low alcoholic self carbonated fermented beverage. *Int J Food Res* **17**:969-76.

Sahota P, Kaur D and Pandove G (2010b) Studies on the preparation of low alcoholic naturally carbonated blended beverage from guava and lemon. *Internet J Food Safety* **12**:165-80.

Sahota P, Pandove G, Jairath S and Phutela R P (2009) Preparation of Low alcoholic self carbonated beverages from carrot amla juices. *Bev and Food World* **36**:15-17.

Saitou N and Nei M (1987) The neighbour-joining method a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**:406-25.

Saleem M (1980) *Studies on the preparation of comminuted citrus fruit beverage base*. M.Sc. Thesis, University of Agriculture, Faisalabad, India.

Sambrook J and Russel D W (2001). In "Molcular cloning: a labolatory manual". In: Ford N, Nolan C, Ferguson M and Ockler M (2nd ed) *Cold Spring Harbor laboratory*. Press, New York.

Saranya D, Shanmugam S, Kumar S T , Thayumanavan B and Rajasekaran P (2009) Purification and characterization of naringinase from *Candida tropicalis*. *Advanced Biotech* 11-13.

Sarolia D K and Mukherjee S (2002). Comparative efficiency of different preservation methods in keeping quality of lime (*Citrus aurantifolia*) swingle juice during storage. *Haryana J Horti Sci* **31**:185-88.

Scaroni E, Cuevas C L and Ellenrieder G (2002) Hydrolytic properties of crude alpha-L-rhamnosidases produced by several wild strains of mesophilic fungi. *Lett Appl Microbiol* **34**:461-65.

Scopes R K (1994) *Protein Purification: Principles and Practice*. Pp 146-85. Narosa publishing House, New Delhi.

Sevda S B and Rodrigues L (2011) Fermentative Behavior of Saccharomyces Strains During Guava (*Psidium Guajava* L) Must Fermentation and Optimization of Guava Wine Production. *J Food Process Technol* **2**:41-49.

Shanmugam V and Yadav K D S (1995) Extracellular production of alpha rhamnosidase by *Rhizopus nigricans*. *Ind J Exp Biol* **33**:705-07.

Shaw P E and Wilson C W (1983) Debittering citrus juices with P-cyclodextrin polymer. *J Food Sci* **48**:646.

Singh B P, Gupta A K and Chundawat B S (1978) Effect of various treatments on storage of kinnow fruits. *Punjab Hort J* **18**:61-65.

Singh R K, Kumar D P, Solanki M K, Singh P, Srivastva A K, Kumar S, Kashyap P L, Saxena, A K, Singhal P K and Arora D K (2012) Optimization of media components for chitinase production by chickpea rhizosphere associated *Lysinibacillus fusiformis* B-CM18. *J Basic Microbiol* **52**:1-10.

Singleton V L, Joseph A and Rossi J R J A (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticulture* **16**:144-58.

Sogi D S and Singh S (2001) Studies on bitterness development in kinnow juice ready-to-serve beverage, squash, jam and candy. *J Food Sci Technol* **38**:433-38.

Soria F F, Cuevas C and Ellenrieder G (1999) Purification and some properties of α-L-rhamnosidase of *Aspergillus terreus*. *Appl Biol Sci* **5**:109-20.

Spagna G, Barbagallo R N, Martino A and Pifferi P G (2000) A simple method for purifying glycosidases: α-L-rhamnopyranosidase from *Aspergillus niger* to increase the aroma of Moscato wine. *Enzyme Microb Technol* **27**:522-30.

Sun T, Powers J R and Tang J (2007) Enzyme-catalyzed change of antioxidants content and antioxidant activity of asparagus juice. *J Agric Food Chem* **10**:56-60.

Suzuki H (1962) Hydrolysis of flavonoid glycosides by enzymes (Rhamnodiastase) from Rhamnus and other sources. *Arch Biochem Biophys* **99**:476-83.

Tamura K Dudley J, Nei M and Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**:596-1599.

Terada Y, Kometani T, Nishimura T, Takii H and Okada S (1995) Prevention of hesperidin crystal formation in canned mandarin orange syrup and clarified orange juice by hesperidin glycosides. *Food Sci Technol Int* **1**:29-33.

Thammawat K, Pongtanya P, Juntharasari V and Wongvithoonyaporn P (2008) Isolation, preliminary enzyme characterization and optimization of culture parameters for the production of naringinase isolated from *Aspergillus niger* BCC25166. *Kaestsart J Nat Sci* **42**:61-72.

Thomas D W, Smythe C V and Labbee M D (1958) Enzymatic hydrolysis of naringin, the bitter principle of grapefruit. *Food Res* **23**:591-98.

Ting S V (1958) Enzymatic hydrolysis of naringin in grapefruit. *J Agric Food Chem* **6**:546-49.

Vaks B and Litshitz A (1981) Debittering of orange juice by bacteria which degrade limonin. *J Agric Food Chem* **29**:1258-61.

Van der Walt J P (1999) Interpreting the automictic ascomycetous yeasts. *S Afr J Sci* **95**:440-41.

Van der Walt J P and Von Arx J A (1985) The septal ultrastructure of *Hormoascus ambrosiae* and the emendation of the genus Hormoascus. *Syst Appl Microbiol* **6**:90-92.

Van der Walt J P and Yarrow D (1984) Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij N J W (ed) *The Yeasts: A taxonomic study*. Pp 45-105. Elsevier Science Publishers, Amsterdam.

Veldhius M K (2nd ed) (1971) *Fruit and vegetable juice processing technology*. The Avi Publishing Company, Inc.

Versaslovic J, Schneider M, de Bruijn F J and Lupski J (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Cell Biol* **5**:25-40.

Vila-Real H, Alfaia A J, Calado A R T and Ribeiro M H L (2010) Improvement of activity and stability of soluble and sol-gel immobilized naringinase in co-solvent systems. *J Mol Catal B Enz* **65**:10-91.

Viniegra G, Favela E, Aguilar C N, Romero G, Omez S J, Diaz-Godnez G and Augur C (2003) Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem Eng J* **13**:157-67.

Vishwanatha T, Spoorthi N, Jain R V and Divyashree B C (2010) Screening of substrates for protease production from *Bacillus licheniformis. I J E S T* **2**:6550-54.

Walker G M, Maynard A I and Johns C G W (1990) The importance of magnesium ions in yeast biotechnology. *J Ferment Technol* **28**:233-40.

Wang L, Chi Z, Wang X, Ju L and Chi Z (2008) Isolation and characterization of *Candida membranifaciens* subsp. flavinogenie W143, a novel riboflavin-producing marine yeast. *Microbiol Res* **163**:255-66.

Wood T M and Bhat K M (1988) Methods for measuring cellulase activities. *Methods Enzymol* **160**:87-112.

Yadav S and Yadav K D S (2001) Immobilization of α-L-rhamnosidase by *Aspergillus terreus* MTCC-3374 on bagasse particles based matrix. *Indian J Chem Technol* **8**:314-18.

Yadav S and Yadav K D S (2004) Secretion of *α*-L-Rhamnosidase by some indigenous fungal strains. *J Sci Ind Res* **63**:439-43.

Yadav S, Yadav V, Yadav S and Yadav K D S (2012) Purification, characterization and application of α-L-rhamnosidase from *Penicillium citrinum* MTCC-8897. *Int J Food Sci Technol* **47**:290-98.

Yadav V, Pramod K, Yadav L, Yadav S and Yadav K D S (2010) α-L-Rhamnosidase: A review *Process Biochem* **45**:1226-35.

Yanai T and Sato M (2000) Purification and characterization of α-L-rhamnosidase from *Pichia angusta* X349. *Biosi Biotechnol Biochem* **64**:2179-85.

Yarrow D (1998) Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman C P and Fell J W (ed) *The yeasts, a taxonomic study*. Pp. 77-100. Elsevier, Amsterdam.

Yarrow D (2000) Methods for isolation, maintenance, and identification of yeasts. In: Kurtzman C P and Fell J W (ed) *The yeasts: a Taxonomic Study*. Pp 77-100. Elsevier, Amsterdam.

Young N M, Johnston R A Z, Richards J C (1989) Purification of α-Lrhamnosidase of *Penicillium decumbens* and characterisation of two glycopeptide components.

Carbohydr Res **191**:53-62

Yu H, Gong J, Zhang C and Jin F (2002) Purification and characterization of ginsenoside α-L- rhamnosidase. *Chem Pharm Bull* **52**:175-78.

Zia M A (1987) *Production and characterization of fruit juice blends of mango, pomegranate and guava*. M.Sc. Thesis, University of Agriculture, Faisalabad, India.

Zverlov V V C, Hertel K, Bronnenmeier A, Hroch J, Kellermann and W H Schwarz (2000) The thermostable α-L-rhamnosidase RamA of *Clostridium stercorarium*: biochemical characterization and primary structure of a bacterial α-L-rhamnosidase hydrolase, a new type of inverting glycoside hydrolase. *Mol Microbiol* **35**:173-79.

ANNEXURE

Table I **Saturated ammonium sulphate precipitation tables**

Ammonium sulphate, Grams to be added to 1 L

From S_1% ↘	From S_2% → 5	10	15	20	25	30	35	40	45
0	27	55	84	113	144	176	208	242	277
5		27	56	85	115	146	179	212	246
10			28	57	86	117	149	182	216
15				28	58	88	119	151	185
20					29	59	89	121	154
25						29	60	91	123
30							30	61	92
35								30	62
40									31
45									
50									

From S_1%	50	55	60	65	70	75	80	85	90	95	100
0	314	351	390	430	472	516	561	608	657	708	761
5	282	319	357	397	439	481	526	572	621	671	723
10	251	287	325	364	405	447	491	537	584	634	685
15	219	255	292	331	371	413	456	501	548	596	647
20	188	223	260	298	337	378	421	465	511	559	609
25	157	191	227	265	304	344	386	429	475	522	571
30	126	160	195	232	270	309	351	393	438	485	533
35	94	128	163	199	236	275	316	358	402	447	495
40	63	96	130	166	202	241	281	322	365	410	457
45	31	64	97	132	169	206	245	286	329	373	419
50		32	65	99	135	172	210	250	292	335	381
55			33	66	101	138	175	215	256	298	343
60				33	67	103	140	179	219	261	305
65					34	69	105	143	183	224	266
70						34	70	107	146	186	228
75							35	72	110	149	190
80								36	73	112	152
85									37	75	114
90										37	76
95											38

For 1 L of solution: Amount of ammonium sulphate (solid) to be added to a solution already at S_1% saturation to take it to S_2% saturation.

$$\mathbf{g = \frac{533\,(S_2 - S_1)}{100 - 0.3S_2}}$$

Table II Production cost of partial purified enzyme

Sr. No.	Material	Quantity	Price (Rs.)
1.	Yeast culture	0.75% (v/v)	5
2.	Minimal medium	10 g	20
3.	Ammonium sulphate	160 g	45
4.	Dialysis	-	10
Total cost of enzyme produced (30 mL) from 1 litre production media			**80**
***10 mL enzyme is used in 10 litre kinnow juice** **So total cost of enzyme is Rs. 26.66 for 10 litre kinnow juice**			

Table III Production cost for preparation of debitterd low alcoholic naturally carbonated kinnow beverage (LANC)

Sr. No.	Material	Quantity	Price (Rs.)
1.	Yeast culture	0.75% (v/v)	5
2.	Kinnow juice	6 L	60
3.	Sugar	500 g	15
Total cost for preparation of debitterd low alcoholic naturally carbonated kinnow beverage (10 L)			**80**

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