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RESEARCH ARTICLE

Characterization of enzyme naringinase and the production of debittered low alcoholic kinnow (Citrus raticulata blanco) beverage.

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Abstract

Naringin, the bitter flavonone glycoside and primary bitter component in citrus fruit juices can be hydrolysed by naringinase enzyme into tasteless component, naringenin. A rapid detection test for naringinase producing microorganisms using $FeCl_3$ has been developed. Naringin an inducer in kinnow juice, mutated yeast *Clavispora lusitaniae* and induced the production of crude enzyme naringinase with the activity of 0.0135 IU/ml. The parameters optimized for naringinase production were pH (4), temperature (50°C), naringin (0.8%) and rhamnose concentration (0.6%) with high substrate activity K_m value of 1.00 mM and V_{max} value of 28.56 mM. Divalent cations Cu^{2+} and Ca^{2+} competitively inhibited enzyme activity where as Mn^{2+} and Zn^{2+} stimulated enzyme activity of 0.0103 IU/ml and 0.0079 IU/ml, respectively. The debittering of kinnow (*Citrus reticulata blanco*) juice by *Clavispora lusitaniae* mutant prompted for utilization of kinnow juice for the production of low alcoholic naturally carbonated fermented kinnow beverage. Technology for the production of low alcoholic naturally carbonated fermented kinnow beverage with yeast *Clavispora lusitaniae* under optimized fermentation conditions were developed. Microbiological, physiochemical and sensory evaluation of kinnow beverage with 40 percent juice revealed pH 4.2, TSS 10.5°B, acidity 0.53%, ascorbic acid 9.2 mg/100ml, reducing sugar 8.83 percent, total sugars 9.4 percent, limonin 1.7 ppm, naringin 120.8 ppm, β-carotene 0.26µg/l, alcohol 0.28% (w/v), CO_2 1.09 bar and plate count $9.3x10^8$ cfu/ml, ranked highest for taste 7.9, aroma 8.5, colour 7.8, astringency 8.25 and overall acceptability 7.8 during storage period of 70 days under refrigerated conditions (4°C). The percentage decrease in limonin and naringin on storage was 54 and 64.8 percent, below the threshold level of limonin (6 ppm) and naringin (600 ppm) respectively.

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INTRODUCTION

India leads in the production of fruits and stands third in the production of citrus fruits in the world. Kinnow contributes towards major fruit production in Punjab where the total area under fruit cultivation is 0.66 lakh hectares. Of which, 31,800 hectares with the production of 6.00 lakh tonnes comes under kinnow production. NHB (2010) Kinnow mandarian is one of the major citrus fruit crops with superior characteristics such as heavy bearing, wide adaptability, fruit quality, high juice content. The juice has high therapeutic value as antispasmodic, sedative, cytophylactic, digestive, anti carcinogenic, anti inflammatory and anti allergic.

Owing to the post harvest infrastructure over a short period, efficiency marketing and utilization of kinnow gets effected. So, processing of kinnow is necessary to solve the problem of market surplus, related its spoilage. Khandelwal *et al* (2006) The processing of citrus fruits to beverages have encounterd commercial restrictions due to their undesirable bitter taste, which is attributed to the presence of naringin (4,5,7-trihydroxyflavonone-7-rhamnoglucoside), triterpenoid dilactones ("limonoids"), limonin and nomilin (Thammawat *et al* 2008).The intact fruit contains limonin, a non bitter precursor or limonite-A-ring lactone (LARL) originally present in the insoluble form in fruit sections passes into the juice after its preparation and is converted into limonin under acidic conditions. 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).

Naringin level can be reduced by different techniques such as resin absorption Maria *et al* (2002), supercritical carbondioxide extraction, β-cyclodextrin treatment Shaw and Wilson, (1983) and enzymatic hydrolysis. In comparison, the enzymatic debittering technology is the most promising method due to its high specificity, efficiency and convenient procedure for debittering beverages on commercial scale. Yadav *et al* (2010).The biotechnological potential of microbial naringinase has drawn great attention from researchers worldwide for enzymatic debittering of citrus fruit juices. Naringinase is an enzyme complex, containing α-rhamnosidase (E.C. 3.2.1.40) and β-glucosidase. Naringin hydrolysis is a two step reaction where α-rhamnosidase first splits naringin into L-rhamnose and prunin and and β – glucosidase hydrolyses prunin into non bitter naringenin and D-glucose (figure-1).

Naringinase has been reported in plants (cereals, grapefruit leaves), yeasts (*Candida tropicalis*), fungi *(Aspergillus niger*) and bacteria (*Penicillium, Coniothyrium diplodiella*). The microbial naringinase is gaining special attention in the recent days due to its cost efficient production, hence replacing other chemical methods for naringin reduction. Temperature, pH variation, enzyme concentration and inducer concentration are important parameters responsible for stability and activity of an enzyme action.

Naringinase has potential biotechnological applications in citrus processing and bioprocess industries such as wine industries for aroma enhancement, glycolipids production, biotransformation of steroids, antibiotics, and mainly of glycosides hydrolysis. Many natural glycosides, including naringin, rutin, quercitrin, hesperidin, diosgene, and ter-phenyl glycosides, containing terminal α-rhamnose and D-glucose can act as substrates of naringinase. The products of naringinase action exhibits antioxidant, anti-inflamatory, antiulcer, hypocholestrolemic, antimutagenic, nuroprotective and antiviral effects. Ribeiro (2011).

The objective of this study was to characterize enzyme naringinase produced by *Clavispora lusitaniae* with respect to temperature, pH, inducer concentration and to exploit its potential for the development of novel, commercially viable biotechnological process for the preparation of low alcoholic naturally carbonated kinnow beverage.

Material and methods

Yeast culture

The yeast culture, *Clavispora lusitaniae*, was procured from the Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab India.

Vegetative growth in liquid and solid media

Growth of yeast in both liquid and solid media (4% glucose-peptone-yeast extract) was examined for characterization of vegetative morphology such as cultural growth pattern, texture, colour, shine, surface elevation and margin cell shape, mode of reproduction, cell size, surface growth and odour. Identification of yeast isolate on the basis of biochemical and physiological activities included fermentation of sugars.

Vegetative Morphology of yeast in response to inducer naringin

Culture media and growth conditions

Yeast *Clavispora lusitaniae* was grown on modified GYE medium with inducer naringin at ambient pH and temperature to induce the development of vegetative morphotypes.

Screening of yeast capable of producing naringinase

Rapid Detection Test for naringinase producing microorganism

Naringinase producing yeast, *Clavispora lusitaniae* was analysed for naringinase production using 1% ferric chloride without using assay methods. The naringinase producing *Clavispora lusitaniae* was grown on glucose yeast agar containing naringin (1%). If the organism produces naringinase then the naringin present in the medium will be cleaved and the end product naringenin reacts with ferric chloride and produces reddish brown color.

Naringinase assay

Assay of naringinase was carried out by slight modification in the method of Thammawat *et al* (2008) using naringin as substrate. Naringin (0.65 mL of 0.1%) in 0.01 M acetate buffer (pH-4.0) was made to react with 0.1 ml of naringinase enzyme for 15 min at 60°C. From the reaction mixture, 0.2 ml was taken and mixed with 4 ml of 90% diethylene glycol and 0.2 mL of 4N NaOH. This mixture was kept for 10 min at an ambient temperature. The resulted yellow colour was measured at 420 nm. Determination of enzyme activity was calculated using pure naringin as standard. One unit (IU) of naringinase activity was defined as the amount of enzyme that could hydrolyze 1µmol of naringin/ml in min at the assay conditions.

Enzyme Extraction

Enzyme was procured by inoculating GYE broth containing naringin (1%), rhamnose (0.4%), and divalent ion Pb^{2+} (10 mM) with yeast *Clavispora lusitaniae* and incubating it at 37°C for 5 days. Extracellular crude enzyme naringinase was extracted in the supernatant by centrifugating at 4°C at 12,000 rpm for 10 minutes. Supernatant (crude enzyme, naringinase) was collected for its characterization with respect to temperature, pH, inducer concentration and kinetic studies.

Enzyme characterization

The optimum pH for naringinase was determined by pre-incubating the enzyme at 50°C in sodium acetate buffers at the pH range from 2.5 to 6 and measuring the activity using assay method. The temperature optima was assessed by preincubating the enzyme at optimum pH 4 at various temperatures (5°C to 70 °C) and following the enzyme activity.. The Michaelis Menten constants (Km and Vmax) were determined by non-linear regression at concentrations ranging from 0.1 to 0.5 µmoles. Substrate specificity studies were performed using L-rhamnose (inducer) and naringin (inducer and substrate) in the culture media at concentrations ranging from 0.2% to 1% following naringinase activity by assay method.

Effect of metal ions

Various divalent metal ions ($Fe^{2+,}$ Cu^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} , Zn^{2+}, Hg^{2+}, Pb^{2+}) were incorporated at concentration10 mM to the culture media and their effect on the production of naringinase was studied by determining naringinase activity.

Preparation of low alcoholic naturally carbonated kinnow beverage to investigate debittering capability of *Clavispora lusitaniae* producing enzyme naringinase.

Selection of Fruits

Kinnow (*Citrus reticulata Blanco*) was procured from Department of Horticulture, PAU, Ludhiana, Punjab, India. Healthy fruits were selected after manually sorting and discarding defective fruits. Fruits were washed in chlorinated water and then used for the extraction of juice. Juice was extracted aseptically under hygienic conditions.

Extraction of juice

Healthy even sized fruits were selected. These were washed, calyx removed and then cut into two pieces. Juice was extracted by kinnow juice extractor. Extracted juice was filtered through muslin cloth.

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. Brix of diluted juice was adjusted to 14ºB by adding sugar solution and palatable acidity varying from 0.32-0.40%.

Inoculum preparation

The inoculum was prepared in diluted juice with brix adjusted to (13 °Brix). A loopful culture of 24 h old yeast *Clavispora lusitaniae* culture was inoculated in 100 ml diluted kinnow juice in 250 ml Erlenmeyer flask and incubated at 37^0 C for 24 hours to achieve concentration of 10^5-10^6 cells/ml.

Preparation of sugar solution

Granulated sucrose was procured from local market. The sugar solution was prepared by boiling (500g) 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.

Fermentation

The diluted juice was inoculated @ 0.5% v/v with freshly prepared inoculum and incubated at 30±5^{0}C for 36 hours in batch scale glass digester.

Bottling and shelf life studies

The beverage was refrigerated for 24 hours, siphoned, bottled and then stored in refrigerated conditions (4°C). Shelf life of non-alcoholic naturally carbonated fermented kinnow beverage was studied and evaluated fortnightly for physicochemical, microbiological and sensory qualities.

Naringin estimation

Naringin was estimated by Davis method, (1947) Standard solutions of naringin (1000 ppm stock solution) was prepared by dissolving 100 mg of naringin in 100 ml of warm distilled water. Diluted with distilled water to make 100, 200, 300, 400, 500, and 600 ppm standard solutions. 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 minutes, 0.5 ml of 4N NaOH was then added and again mixed well. It was left undisturbed for 15 minutes. The optical density of resulted yellow color was measured at 420 nm.

Results and Discussion

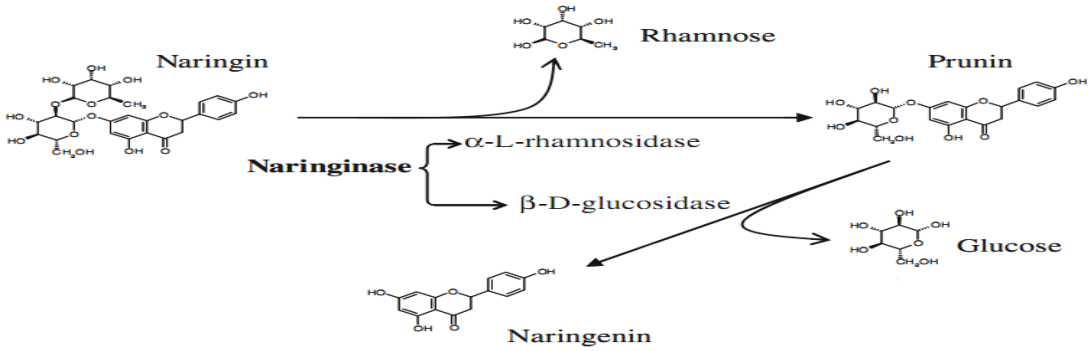


Figure 1 Hydrolysis of naringin into prunin, rhamnose, naringenin and glucose by naringinase containing -l-rhamnosidase and β-d-glucosidase activities.

Morphological, physiological and biochemical characterization of *Clavispora lusitaniae* EF221824

Clavispora lusitaniae EF221824 exhibited viscous texture with off-white colouration and a matt appearance on solid media (GYE). After 24 hours of incubation of yeast in GYE broth at 30°C, the cells exhibited budding and were mostly elliptical. The optimum pH and temperature range for yeast *Clavispora lusitaniae* is 3.0-5.0 and can grow and ferment at wide temperature range of 10-40°C. Yeast was able to assimilate dextrose, fructose, sucrose, maltose, raffinose, cellobiose, lactose, mannose, galactose, inositol, mannitol, trehalose and salicin. The results of the carbon assimilation are summarized in Table 1. This significant property of yeast *Clavispora lusitaniae* to ferment wide range of sugars, makes it a potential source for its utility to ferment wide range of fruit juices for the production of low alcoholic naturally carbonated beverages.

Complex Colony Morphotypes in response to inducer naringin

Yeast *Clavispora lusitaniae* EF221824, is most often described as a simple unicellular organism. Despite this perception *Clavispora lusitaniae* displayed a surprising array of behaviors, involving complex interactions between cells. Under nutrient rich conditions, *C. lusitaniae* grow via ‘‘yeast form,’’ mitotic growth, rapidly dividing and forming smooth, round colonies on solid media. Limitation of one or more key nutrients or the presence of inducer triggered a variety of developmental responses. *Clavispora Lusitaniae* (EF221824) exhibited mutagenesis with inducer naringin (0.2%), followed by plating on solid media yielded colony morphology variants characterized

by raised, rough-surfaced colony of irregular outline in marked (Plate-2) contrast to the flat, shiny, circular colonies of the parental strain (Plate-1). The pleomorphic form of *Clavispora lusitaniae* i.e. yeast germ tube pseudohyphae and true hyphae developed in response to nutritional inducer naringin over a wide temperature and pH range of 10-60°C and 2-8 respectively. Altered pattern of carbohydrate assimilation of mutant to that of parent strain have been reported. But the mutant was able to assimilate fructose, maltose, dextrose, trehalose, sucrose same as that of parental strain.The results also showed that induction of colony morphology is primarily carbon source dependent with the strongest effects induced by reduced dextrose (1% dextrose w/v). Increasing dextrose concentration (4% dextrose YPD) inhibits the colony morphology response, providing further evidence that carbon source limitation is also a trigger for complex colony morphology. A similar behavior, had been observed by Gagiano *et al* (2002) and Pan *et al* (2000) in haploid cells of *S. cerevisiae* grown under dextrose limitation or in the presence of various alcohols . Nitrogen starvation combined with a non-fermentable carbon source induces sporulation, meiosis and pseudohyphal growth, which is characterized by elongated cells, agar invasion and unipolar budding, where mother and daughter cells remain attached.

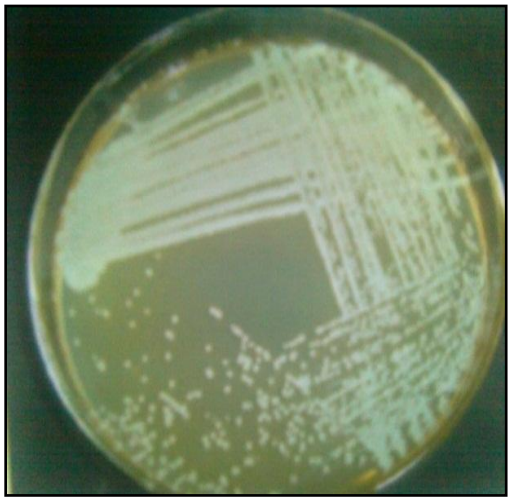


Plate-1 *Clavispora lusitaniae* (EF221824)

Plate-2 Morphotype of mutant *Clavispora lusitaniae*

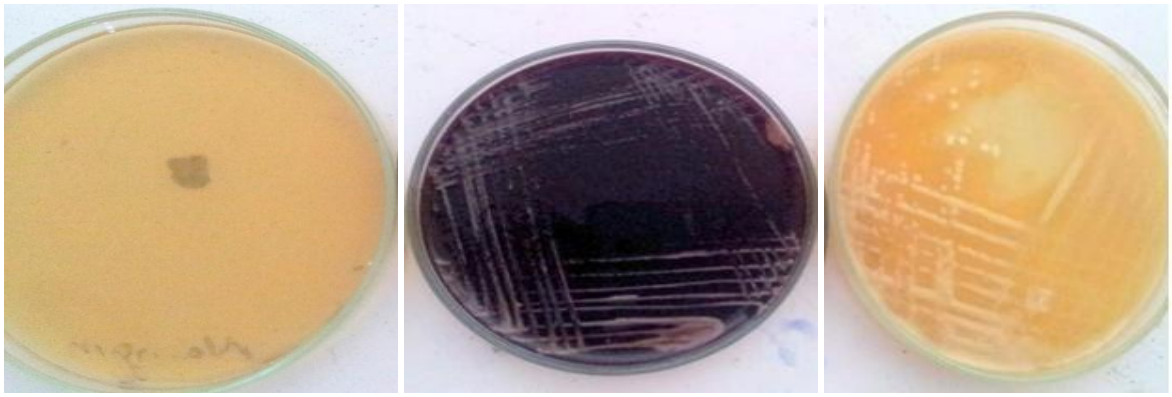
Table 1: Characteristics of yeast *Clavispora lusitaniae* and its mutant based on carbohydrate fermentation

Carbohydrates	*Clavispora lusitaniae*	*Clavispora lusitaniae* mutant
Cellobiose	+	–
Raffinose	+	–
Lactose	+	–
Adonitol	–	–
Dulcitol	–	–
Fructose	+	+
Maltose	+	+
Mannose	+	–
Galactose	+	–
Dextrose	+	+
Sorbitol	–	–
Inulin	–	–
Rhamnose	–	–
Xylose	–	–
Arabinose	–	–
Inositol	+	–
Mannitol	+	–
Melibiose	–	–
Trehalose	+	+
Salicin	+	–
Sucrose	+	+

Screening of yeast capable of producing naringinase

Rapid Detection Test for naringinase producing microorganism

The study investigated the rapid detection method of naringinase producing microorganism by pouring 1% (v/v) ferric chloride solution directly over the petriplate (Figure 2). Ferric chloride reacted with naringin to give reddish brown color naringenin, the end product of naringinase action on naringin. So, reddish brown colour acquired by the yeast *Clavispora lusitaniae* is the positive test for naringinase. This test was performed again with the end product for the confirmation of the above results. Radhakrishnan *et al* (2013) also reported the test for screening the naringinase producing isolate using $FeCl_2$ for *Aspergillus flavus* for debittering of citrus fruit juices.



a **b** **c**

Figure: 2 Rapid Detection Test for naringinase producing microorganism using ferric chloride A: control plate with ferric chloride, b: organism producing naringinase, *Clavispora lusitaniae* c: organism not producing naringinase.

Characterization of crude naringinase enzyme

Effect of pH

The dependence on the pH of naringinase from *C.lusitaniae* was determined according to its naringinase activity. The enzyme activity at pH 2.5, 3, 4, 5 and 6 were found to be 0.04 , 0.006, 0.0224, 0.0144 and 0.008(IU/ml), respectively (figure-3).The results revealed that maximum naringinase activity of 0.0224IU/ml was at pH 4. So, optimum pH for activity of enzyme naringinase is 4. The possible reason for decrease in enzyme activity above and below the pH 4 may be the variation in pH value above or below its optima can irreversibly change in enzymatic structure by altering charge of amino acids responsible for secondary and tertiary structure and it may also depend upon microbial sources which prevail at different pH optoma range of pH 4.0 to 6.5. The high response at low pH level, is of great importance in fruit juice processing industry because pH of juices is often less than 5. This depicts that enzyme preparation will work under optimum pH conditions, thus increasing operational stability (Busto *et al* 2007). 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. Suggesting no need for addition of preservatives in the beverage to increase its shelf life. Thus, this potential of enzyme can be utilized for the prepration of fruit beverages without preservatives

The optimum pH 4 of *C.lusitaniae* is corroborates with that reported for *Candida tropicalis* Saranya *et al* (2009), *Aspergillus niger* 1344 Puri and Kalra (2005), *A. niger* BCC 25166 Thammawat *et al* (2008) and lower than that reported for *Aspergillus flavus* Radhakrishnan *et al* (2013) with pH optima of 3.0

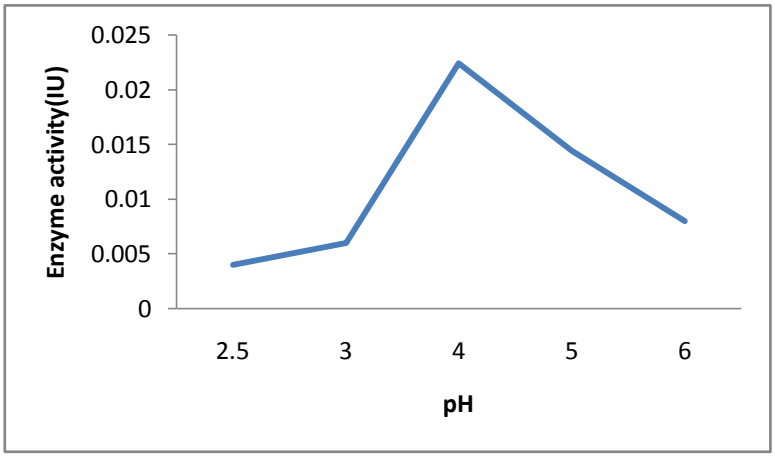


Figure 3 Optimum pH of enzyme naringinase

Effect of temperature

The optimum temperature is determined by the balance between the effect of temperature on the rate of enzyme reaction and its effect on the rate of distortion of the enzyme structure. Busto *et al* (2007). There was slight increase in enzyme activity from 0.00023 to 0.0023 IU/ml at temperature 5°C and 15°C, respectively followed by rapid increase in enzyme activity from 0.0099 to 0.0158 at temperature 30 and 40°C, respectively. Maximum enzyme activity (0.0224 IU/ml) was observed at the temperature range from 40 to 50°C followed by exponential decrease in enzyme activity (0.046 to 0.00354 IU/ml), with increase in temperature from 60°C to 70°C (Figure 4). The results revealed that the maximum naringinase activity of 0.0224 IU/ml at temperature 50°C. The reason for the decrease in enzyme activity above and below the optimum temperature may the deactivation of enzyme by weakening 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 50°C. However, optimum temperature reported for *Aspergillus niger* CECT 2008, Busto *et al* (2007) *Candida tropicalis*, Saranya *et al* (2009) and *A. Niger*, Puri and Kalra (2005),were 60°C, 25°C and 50°C respectively.

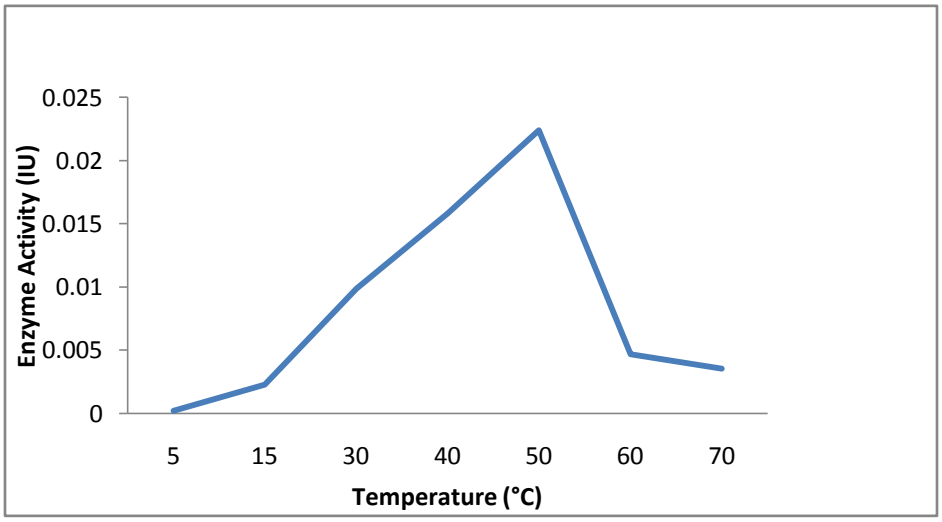


Figure 4 Optimum temperature of enzyme naringinase

Kinetic parameters

The Michaelis constant Km and Vmax value of *C.lusitaniae* naringinase using naringin as substrate were 1mM and 28.56 Mm, respectively (figure-5). High Km value suggests higher reaction rate, since the substrate can outcompete for the binding sites whereas Vmax reveals turnover number of enzyme i.e. number of substrate molecules being catalyse per second. High Vmax value reveals high enzyme activity, suggesting utilization of yeast *Clavispora lusitaniae* for debittering citrus fruit juices. Lower Km values 0.64 mM was reported by Ono *et al* (1978) and higher Km value of 3.6 mM and 1.22mM value was reported by Soares and Hotchkiss and Sekeroglu (2006), respectively. The V_{max} values reported by *Aspergillus niger* (Puri and Kalra 2005) and *Candida tropicalis*, Saranya *et al* (2009) was 21U/mg and 0.1 U/mg, respectively.

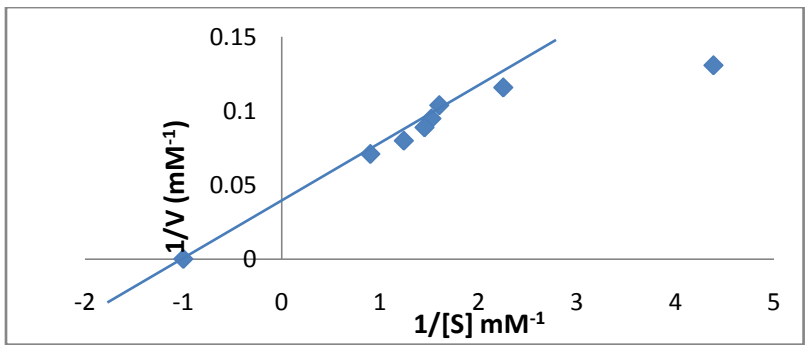


Figure 5 Lineweaver Burk plot of effect of naringin concentration on enzyme naringinase. Reaction conditions: 0.2 ml enzyme ; 0.1M sodium acetate buffer; pH: 4.0; temperature: 50°C; reaction time: 50 minutes.

Effect of various substrate and inducer concentrations on naringinase production:

Clavispora lusitaniae was grown on modified Glucose Yeast Agar medium with different naringin concentrations and rhamnose concentrations (0.2-1%) for enzyme production following enzyme assay method. The enzyme activity at naringin concentrations of 0.2%, 0.4% and 0.6% showed slight increase in enzyme activity of 0.0054, 0.0063, 0.0073 (IU/ ml), respectively followed by rapid increase at 0.8% with enzyme activity of 0.0243 IU/ml. This drastic increase in enzyme activity may be due to increase in the metabolic activity of an enzyme either by binding of inducer to the enzyme and activating it, or by increasing the expression of the gene coding for the enzyme. At 1.0% naringin concentration, naringinase activity showed sudden decrease to 0.0205 IU/ ml, suggesting the inhibition action of high concentration of naringin by naringinase (figure-6). Results revealed maximum activity of 0.0243 IU/ ml at naringin concentration of 0.8%. The enzyme activity at various rhamnose concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% were found to be 0.0067, 0.0071, 0.0051, 0.0054 and 0.0058(IU/ml), respectively. Results revealed maximum enzyme activity of 0.00713IU ml^{-1} at 0.4% of rhamnose concentration (figire-6).On compairing enzyme activities of inducers naringin and rhamnose, maximum activity of 0.0243 and 0.00713 (IU/ ml), respectively were found, suggesting that naringin is better inducer than rhamnose.Kumar (2010) reported naringinase activity in five different medium and maximum naringinase was produced in naringin (7.48 IU/ml) followed by rhamnose (6.72 IU/ml), rutin (3.71 IU/ml), naringenin (3.36 IU/ml) and hesperidin (2.11 IU/ml). Among the substrates, naringin act as a selective inducer, because it induces faster and maximized naringinase production.

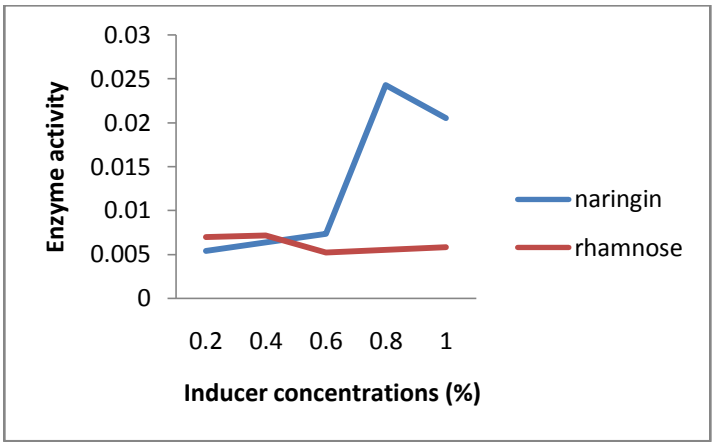


Figure: 6 Effect of naringin and rhamnose at concentrations (0.2-1.0%) for naringinase activity

Effect of metal ions

Various divalent metal ions ($Fe^{2+,}$ Cu^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} , Zn^{2+}, Hg^{2+}, Pb^{2+}) were incorporated at concentration 10 mM to determine their effects on naringinase production by *Clavispora lusitaniae*. Metal ions $Fe^{2+,}$ Cu^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} , Zn^{2+}, $Hg^{2+}$and Pb^{2+}showed enzyme activity of 0.0103, 0.00418, 0.0129, 0.00329, 0.00532, 0.0135and 0.0079(IU/ml), respectively. Results revealed that Fe^{2+}, Cu^{2+} and Ca^{2}show an inhibitory action on the enzyme production by *Clavispora lusitaniae*. Pb^{2+} exhibited the maximum activity 0.01355 IU/ml followed by Hg^{2+} with enzyme activity 0.01292 IU/ ml at 10 mM. Mn^{2+} and Zn^{2+} showed significant enzyme activity of 0.01039 IU ml^{-1} and 0.00798IU ml^{-1} respectively (Figure 8).The reason for increase in enzyme activity by incorporating metal ions may be that the metals get tightly bound to the enzyme along with the substrate, can participate in catalytic reaction in several ways. Ionic interactions between an enzyme-bound metal and a substrate may help orient the substrate for reaction or stabilize charged reaction transition states. Metals can also mediate oxidation-reduction reactions by reversible changes in the metal ion's oxidation state.

Citrus fruits also contains electrolytes; sodium (3-4mg/ml), potassium (300mg in 178ml juice); minerals: calcium (40mg), copper (39mg), iron (0.1mg), magnesium (10mg), manganese (0.024mg) and zinc (0.8mg) per 100g (USDA National Nutrient Database) which may stimulate naringinase production by *Clavispora lusitaniae* during the production of low alcoholic naturally carbonated beverage.

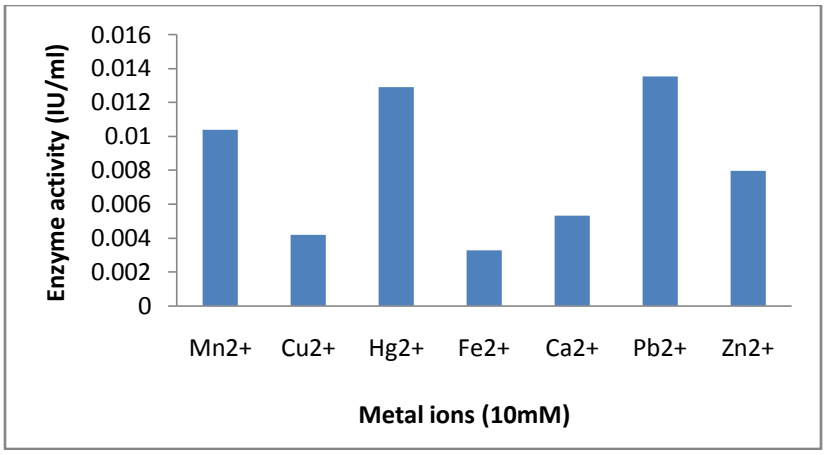


Figure 8 Effect of metal ions on naringinase activity

High efficiency of *Clavispora lusitaniae* for debittering low alcoholic naturally carbonated kinnow beverage.

Low alcoholic naturally carbonated kinnow beverage was prepared with 13ºB by adding and palatable acidity varying from 0.32-0.40%. The diluted juice was inoculated @ 0.5% v/v with freshly prepared inoculum of *Clavispora lusitaniae* and incubated at 15±5^{0}C for 36 hours for fermentation. Prepared beverage was stored at refrigerated temperature (4^{0}C). Limonin and naringin content of beverage was estimated at first and last day of the storage study to investigate the debittering efficiency of yeast *Clavispora lusitaniae* and the limonin and naringin content was found to be much below the threshold level. The threshold level of limonin and naringin in kinnow juice was found to be 6 ppm and 600 ppm respectively.A gradual decrease in limonin with storage decreased from 7.6 ppm to 4.0 ppm (figure-9) while the decrease in naringinwas from 182.383 ppm to 136.787ppm was found (figure-10). The reason for the gradual decrease in the naringin and limonin content may be the production of enzyme naringinase by yeast *Clavispora lusitaniae* leading to the naringin hydrolysis into naringenin (tasteless component).Sharma S (2012) reported the percentage decrease in limonin with storage was 54 percent as it decreases from 7.2 ppm to 3.7 ppm while the percent decrease for the naringin was 64.8 percent as it decrease gradually from 460.2 to 161.9 ppm after 90 days.

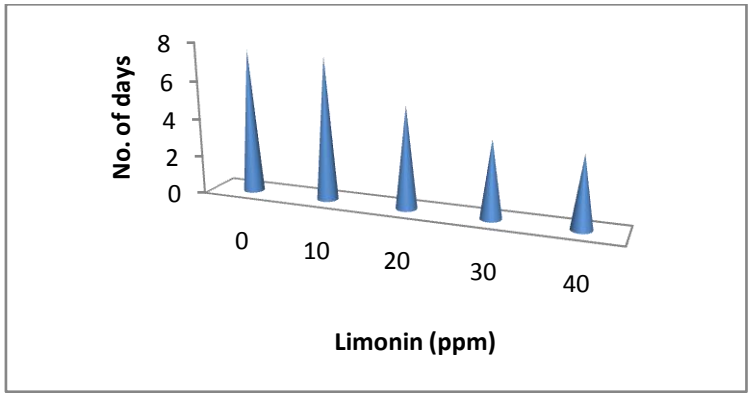


Figure:9 Effect of storage time on limonin concentration in kinnow beverage

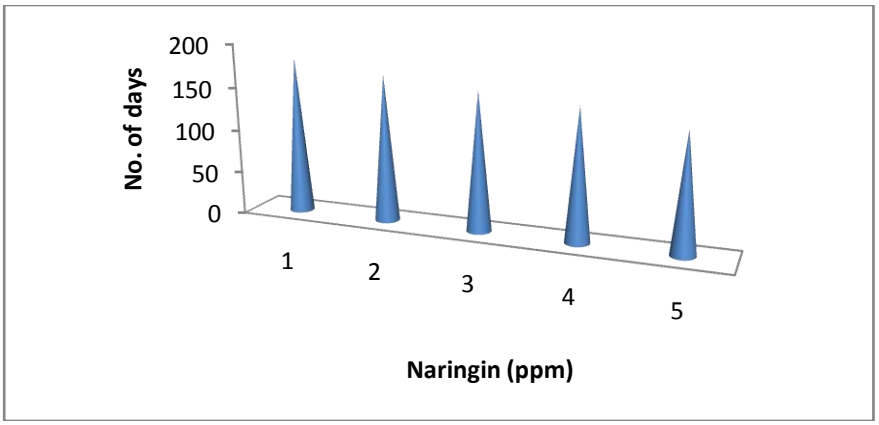


Figure:10 Effect of storage time on naringin concentration in kinnow beverage

Physicochemical analysis raw kinnow juice and low alcoholic naturally carbonated kinnow beverage.

Physicochemical Parameters	Raw kinnow juice	Low alcoholic naturally carbonated kinnow beverage
pH	3.9	3.3
TSS (°Brix)	10.1	11.2
Titrable acidity (% lactic acid)	0.28	0.28
Brix-acid ratio	32.14	40.0
Total Sugars (mg/100ml)	9.5	10.72
Ascorbic acid (mg/100ml)	33.4	24.8
Reducing sugars (%)	2.2	0.720
Naringin (ppm)	182.38	169.43
Limonin (ppm)	7.6	7.2
Beta-carotene (µg/l)	0.327	0.46
Alcohol (%, v/v)	-	0.11
CO_2 (bar)	-	0.7
Total yeast count (cfu/ml)	-	1.11×10^9

Conclusion

The yeast, *Clavispora lusitaniae* was used for the production of enzyme naringinase. This study is worth attempt as the kinetic properties and enzyme characteristics suggests its possible application for the production of debitterd low alcoholic naturally carbonated beverages and aroma enhancement during wine making which is an economical, simple and biotechnological process.

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