Int.J.Curr.Microbiol.App.Sci (2018) *7*(1): 1180-1186

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 01 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.701.143

Enzymatic Production of Debittered Kinnow Juice and Beverage

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Keywords

Kinnow, Yeast, *Clavispora lusitaniae*, Debittering enzymes, Beverage

Article Info

Accepted:
10 December 2017
Available Online:
10 January 2018

A B S T R A C T

The processing of kinnow juice has faced formidable problems in terms of bitterness due to limonin and naringin content thereby affecting its consumer acceptability. To redress this issue, the present study was conducted using fermentative yeast (*Clavispora lusitaniae*) which produces inducible enzymes, naringinase, limonin dehydrogenase, β-glucosidase in kinnow juice. The two technologies optimized are: bioprocess for low alcoholic naturally carbonated beverage and debittering of juice by partial purified enzymes. The physicochemical changes of all the treatments recorded for 30 days of storage period revealed acidity 0.43%, total sugars 52.87 µg/ml, ascorbic acid 32.57 mg/100 ml. The maximum decrease of limonin and naringin content observed in T1 (beverage) with storage was 73% and 79% respectively. The production of kinnow beverage using yeast *C. lusitaniae* found to out compete the effectiveness of the cocktail of debittering enzymes as it is more feasible and economical and can be used further for the commercial purposes. This technology can redress the problem by exploiting the ability of yeast to produce low alcoholic naturally carbonated beverage from nutritive fruits thus making the fruit available throughout the year in the form of beverage.

Introduction

Kinnow (*Citrus nobilis* × *Citrus deliciosa*) is considered as an important fruit of North India. The kinnow fruit cover an area of 3.61 $\times 10^5$ ha and accounts for highest production among all citrus fruits (Anon 2016). The health benefits of kinnow fruit are attributed to the presence of bioactive and antioxidant compounds such as ascorbic acid (53mg), flavonoids, limonoids, coumarins and essential vitamins: folates (30µg), niacin (0.282mg), pantothenic acid (0.25mg), riboflavin (0.040mg), thiamine (0.1mg), Vitamin A (225 IU), Vitamin E (0.18mg) per 100g. Kinnow mandarian juice has high therapeutic value as antispasmodic, sedative, cytophylactic, digestive, anti-carcinogenic, anti-inflammatory and anti-allergic.

However, the processing of kinnow juice had faced commercial restrictions due to inherent bitter taste by chemical limonin (limonoid), naringin (flavonoid) (Thammawat *et al.*, 2008). 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 (Guadagni *et al.*, 1973). Debittering of kinnow juice is an important step for controlling

quality and improving commercial value. Adsorption techniques and enzymatic hydrolysis method have been reported to achieve the goal of debittering of juice.

However, because of the loss of acidity, sweetness flavour and turbidity as well as less efficiency in adsorptive debittering, enzymatic enzymes have showed superior potential in industrial application. To redress the problem of bitterness in citrus juice researchable issues are to device an enzymatic intervention. The juice treated with cocktail of debittering enzymes, produced using novel yeast isolate *Clavispora lusitaniae* mutant, has potential to debitter citrus juices. The debittering enzymes can offer advantages such as cost effectiveness, single step hydrolysis, short incubation, preservation of flavour, retention of color, vitamins and organoleptic components of juice.

The characterized strain has the potential to produce debittering enzymes: Naringinase and Limonin dehydrogenase. Naringinase which hydrolyzes α-L-rhamnosidase to rhamnose and prunin and then by β-glucosidase to glucose and naringenin. Limonin dehydrogenase is an extracellular enzyme, detected in various species of bacteria, that catalyzes the oxidation of Limonoate – A-ring lactone (LARL) (precursor of limonin bound to albedo layer of the fruit at neutral to slightly alkaline pH) to the 17-dehydroxylimonoate (a non-bitter derivative) and prevent limonin (intensely bitter triterpenoid dilactone) production. This enzyme has been reported from bacteria only. The β-glucosidase enzyme catalyzes the hydrolysis of β-glucosidic bond and release D-glucose from non – reducing ends of cellobiose and oligosaccharides and enhances aroma and flavour in citrus juices.

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Materials and Methods

Extraction of juice

Fresh, fully ripened kinnow (*Citrus reticulata* Blanco) fruits were procured from the Department of Fruit Science, Punjab Agricultural University, Ludhiana. The selected fruits were washed in the chlorinated water and then used for the extraction of juice. The juice was extracted aseptically under hygienic conditions with the juice extractor.

Yeast culture

The yeast *Clavispora lusitaniae* (KF 633446) strain producing fermentative debittering enzymes, naringinase, limonin dehydrogenase, β-glucosidase enzymes was obtained from the Department of Microbiology, Punjab Agricultural University, Ludhiana. The strain showed the potential to produce low alcohol (<1%) with a carbonation of 1.5 bars.

The kinnow juice was diluted with water (1:3) and the sugar solution was added to achieve the required TSS 13ºB. The experiment comprised of four treatments consisting of control (unamended) juice, juice inoculated with the yeast (*C. lusitaniae*), juice inoculated with the partially purified debittering enzymes and combination of both. The details of various combinations of treatments are presented in Table 1.

The physico-chemical analysis of initial juice content (acidity, total sugars, ascorbic acid,

limonin, naringin) of fresh kinnow juice was performed.

Shelf life determination

Shelf life of all the treatments was studied and evaluated fortnightly for physicochemical and sensory qualities.

Physicochemical and organoleptic anaylsis

The pH and total soluble solids (TSS) of kinnow juice and beverage were determined using a digital pH meter(ECIL, Hyderabad, type 101) and Erma hand refractrometer of 0-32ºB (Erma,Tokyo, Japan).Total acidity expressed as citric acid was estimated following the procedure of AOAC (1999). Total sugars were estimated by phenol-sulphuric acid method (Dubois *et al.,* 1956). Reducing sugars were estimated by the method (Miller 1959). The titration method using 2,6-dicholorophenol indophenol dye was used to estimate ascorbic acid (AOVC 1996). The content of naringin was estimated by Davis method (1947) and the limonin content was estimated by the colourimetric method (Vaks and Lifshitz 1981).

Results and Discussion

Physicochemical characteristics of kinnow juice (*Citrus reticulata* Blanco)

The physicochemical characteristics of kinnow juice was evaluated on the basis of chemical analysis. The results are presented in the Table 2, showing TSS- 8.00±1.00ºB, pH 4.0, limonin 7.50 ±0.30ppm, naringin 249.75±0.30ppm, reducing sugar 2.54 ±0.02µg/mL, titrable acidity 0.245±0.003%, total sugars 45.25±0.03µg/mL and ascorbic acid 40.55 ± 0.02mg/100mL. In kinnow fruit, the juice content was found to be in the range of 36.00% to 62.00% (Jagjiwan, 2001) and ascorbic acid content in the range of 13.30 to 46.90 mg/100mL (Pruthi *et al.,* 1983; Singh *et al.,* 1978). The acidity and pH of kinnow juice was reported in the range of 0.28-0.51%, 4.20-4.28 by Kaur (2002). The acceptability of beverages is very much dependent on its physicochemical properties including acidity, sugar content, appearance and flavour. There may be changes in the physicochemical characteristics and loss of some compounds that impart flavour and aroma to the beverages during storage (Jairath *et al.,* 2012).

Effect of storage time on physicochemical properties on different treatments

The results of physicochemical properties of kinnow beverage during storage period of 30 days are summarized in Table 3.

The percentage decrease in the limonin content was observed in all the treatments, except control in which the content get raised 5 times higher as compared to the initial content, i.e. 7.5 ppm. The limonin content decreased upto 73.61% in T1 as it decreased from 7.5 ppm to 4.32 ppm followed by the juice with admixture of debittering enzymes i.e. 37.61%. The gradual increase in limonin content is due to conversion of a limonoate – A-ring-lactone (non-bitter) into limonin bitter compound under the acidic conditions of juice (Premi *et al.,* 1994). The limonin content decreased significantly in beverage due to the efficiency of *Clavispora lusitaniae* to metabolize and eliminate the bitter component. Similar results were reported by Singh *et al.,* (2015), Sahota *et al., (*2015) reported that limonin content decreased from 7.6 ppm to 4.0 ppm in kinnow beverage.

The increase in naringin content was observed in control juice from 249.75 ppm to 415.02 ppm during the storage period of 30 days. The decrease of naringin with storage was 249.7 5ppm to 128.18 ppm in T1 is due to the hydrolysis of naringin into rhamnose and

prunin by yeast. The reduction in the naringin content was also observed in the T2 and T3 treatments due to the stability of partially purified debittering enzymes. The results are in accordance with Pandove *et al.,* (2016) observed decrease in naringin content from 410.50ppm to 84.86ppm,Sahota *et al.,* (2015) showed from 182.38 ppm to 136.78 ppm, Singh *et al.,* (2015) showed decrease from in *Citrus reticulata* beverage. The reduction in the reducing sugars content was found in all treatments during the storage period of 30 days. The decrease in glucose in T1 (beverage) was from 2.54µg/mL to 1.97µg/mL due to the utilization of glucose by yeast during fermentation. The percent decrease in T2 was 51.19%.The results found to be accordance with Sahota *et al.,* (2010) who observed decrease in glucose from 7.45 % to 4.82% in fermented blended Luchnow -49 – Baramasi beverage (1:1), Pandove *et al.,* (2016) observed decrease in naringin content from 3.24% to 2.64% in *Citrus reticulata* beverage.

Table.1 Detail of the treatment combinations

Symbols	Treatment Combinations
T0	Control (1:3 kinnow juice)
T1	T0 + *C. lusitanae* (0.75%)
T2	T0 + Naringinase (0.6ml/100ml), Limonin dehydrogenase (0.8ml/100ml), β-glucosidase (0.4ml/100ml)
T3	T0 + T1+ T2

Table.2 Physicochemical characteristics of kinnow juice

Parameters	Kinnow juice
TSS (°B)	8.00±1.00
pH	4.0
Limonin (ppm)	7.50± 0.30
Naringin (ppm)	249.75 ± 0.30
Reducing Sugars (µg/mL)	2.54 ± 0.02
Titrable acidity (%)	0.245 ± 0.003
Total sugars (µg/mL)	45.25 ±0.03
Ascorbic acid (mg/100mL)	40.55± 0.02
Juice Yield (%)	55.00±5.00

Mean value ± standard error of three independent experiments

Table.3 Effect of storage time on physicochemical attributes of kinnow juice and beverage

		Days						
Parameters	Treatment	0	5	10	15	20	25	30
Limonin content (ppm)	**T0**	7.5±0.2	15.68±0.01	25.04±0.04	28.46±0.03	30.64±0.04	33.48±0.01	35.34±0.02
	T1	7.5±0.2	7.68±0.01	6.88±0.01	6.65±0.03	6.57±0.02	6.38±0.01	6.12±0.02
	T2	7.5±0.4	7.14±0.02	6.23±0.02	6.08±0.01	5.78±0.01	5.65±0.04	5.45±0.02
	T3	7.5±0.4	6.68±0.01	5.65±0.04	5.48±0.01	4.86±0.03	4.55±0.04	4.32±0.02
Naringin content (ppm)	T0	249.75±0.01	289.63±0.03	359.22±0.20	348.21±0.01	384.45±0.04	398.12±0.02	415.02±0.02
	T1	249.75±0.02	204.74±0.04	176.76±0.02	166.84±0.04	156.83±0.03	142.53±0.03	128.18±0.01
	T2	249.75±0.02	204.05±0.03	172.42±0.02	168.25±0.04	158.45±0.02	152.36±0.03	148.44±0.04
	T3	249.75±0.01	214.52±0.02	178.95±0.04	167.83±0.02	157.84±0.03	147.95±0.04	139.28±0.01
Glucose content (µg/mL)	**T0**	2.54±0.01	2.18±0.01	1.78±0.01	1.74±0.04	1.67±0.02	1.63±0.03	1.57±0.02
	T1	2.54±0.02	2.57±0.01	2.22±0.02	2.18±0.01	2.12±0.02	2.04±0.04	1.97±0.02
	T2	2.54±0.03	2.12±0.02	1.76±0.03	1.72±0.01	1.62±0.02	1.52±0.02	1.48±0.01
	T3	2.54±0.01	2.28±0.01	1.88±0.01	1.82±0.02	1.75±0.03	1.72±0.02	1.68±0.01
Total Sugars content (mg/mL)	T0	45.25±0.02	46.88±0.01	49.55±0.04	49.78±0.01	52.54±0.04	52.67±0.01	52.84±0.04
	T1	45.25±0.03	46.82±0.02	48.76±0.03	49.67±0.02	52.12±0.02	52.87±0.01	53.07±0.01
	T2	45.25±0.01	45.22±0.02	46.25±0.03	46.54±0.04	46.72±0.02	46.85±0.03	46.98±0.01
	T3	45.25±0.04	45.85±0.03	48.27±0.02	48.65±0.03	48.88±0.01	49.32±0.02	49.57±0.02
Titrable Acidity (%)	T0	0.245±0.003	0.294±0.004	0.375±0.002	0.395±0.004	0.421±0.001	0.442±0.002	0.456±0.003
	T1	0.245±0.003	0.304±0.002	0.384±0.004	0.396±0.003	0.418±0.001	0.426±0.003	0.438±0.001
	T2	0.245±0.001	0.282±0.002	0.362±0.002	0.376±0.003	0.384±0.004	0.425±0.003	0.442±0.001
	T3	0.245±0.002	0.294±0.003	0.374±0.004	0.392±0.001	0.408±0.001	0.422±0.002	0.434±0.004
Ascorbic acid content (mg/mL)	T0	40.55±0.02	28.14±0.04	25.98±0.01	25.54±0.04	25.36±0.02	25.28±0.01	25.08±0.01
	T1	40.55±0.01	36.83±0.02	34.88±0.02	33.85±0.04	33.38±0.01	33.05±0.03	32.57±0.02
	T2	40.55±0.03	28.04±0.04	25.36±0.02	25.08±0.01	24.78±0.01	24.54±0.02	24.38±0.01
	T3	40.55±0.04	35.24±0.03	34.18±0.01	33.28±0.02	33.32±0.01	32.15±0.04	32.02±0.02

Mean Value ± Sum of three independent experiments

T0 = Control, T1 = T0+ *C.lusitaniae*, T2 = T0 + Naringinase (0.6ml/100mL) + Limonin dehydrogenase (0.8ml/100mL) + β-glucosidase (0.4ml/100mL), T3 = T0+ T1+T2

The total sugar content found to increase in all treatments with the advancement of storage period. The maximum increase in total sugars was observed in T2 (beverage) from 45.25µg/mL to 53.07 µg/mL might be due to the hydrolysis of β-glycosidic bond and release of sugar by the β-glucosidase enzyme. The results are in proximation with Dhaka *et al.*, (2016) in kinnow juice, Malav *et al.*, (2014) in orange based RTS beverage.

The increase in acidity in the form of citric acid observed during storage of kinnow juice and beverage along with debittering enzymes. Citric acid is a palatable acidity and remained with the range of consumption and tartness. The percentage increase in acidity was 44.57% that is increase from 0.245% to 0.442% in T2. The percent increase to 44.06% in T1 i.e. from 0.245% to 0.438% and in the T2 the acidity increase to 44.37% and

in kinnow juice the increase was from 0.245% to 0.456% during the storage period of 30 days. The increase in acidity is due to concentration of weakly ionized acid and their salts increased or are due to formation of acid by degradation of polysaccharides.Similar results were reported by Sahota *et al.,* (2010) in blended *Allahabad Safeda – Baramasi* beverage (1:1), Singh *et al., (*2015) in *Citrus reticulata* beverage.

The ascorbic acid content of kinnow juice and beverage along with debittering enzymes decreased with the advancement of storage period because the ascorbic acid is very sensitive to oxygen, light and heat. The percentage decrease to 38.15% in T0, 39.87% in T2. The ascorbic acid decrease to 21.03% in beverage with debittering enzymes. The maximum was in T1 from 40.55 mg/mL to 32.57mg/mL during the period of 30 days. The higher stability was found in beverage which showed its importance in increasing the neutraceutical value over non fermented juice. The CO_2 produced in the beverage forms fizz and displaces the oxygen, hence promoting the stability of ascorbic acid content. The results are in concordance with Dhaka *et al.,* (2016) in kinnow juice, Singh *et al.,* (2015) and Pandove *et al.,* (2016) in *Citrus reticulata* beverage.

The enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency and a convenient method for removing the bitterness. It is concluded that bio-enzymatic debittering by limonin dehydrogenase, naringinase and aroma enhancement by β-glucosidase enzyme produced by *Clavispora lusitaniae* can become the main direction of citrus juices debittering, aromatic and flavour enhancement in future due to its characteristics of high specificity, preferable retention of juice nutrients and economically viable with strong ability to remove the bitter taste and enhance the flavour and aroma in citrus juices which could be stored for 30 days. This technology can redress the problem by exploiting the ability of yeast species to produce low alcoholic naturally carbonated beverage from nutritive fruits thus making the fruit available throughout the year in the form of beverage. The beverage offers advantages like devoid of any chemical preservative, minimally processed, high nutritive value and long shelf life, availability of new formulations and blends, sparkling, tangy taste, effervescent and antimicrobial due to carbonation.

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How to cite this article:

Manmeet Kaur, Parampal Sahota, Neha Sharma, Kirandip Kaur and Bhavish Sood. 2018. Enzymatic Production of Debittered Kinnow Juice and Beverage. *Int.J.Curr.Microbiol.App.Sci.* 7(01): 1180-1186. doi: https://doi.org/10.20546/ijcmas.2018.701.143