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ORIGINAL ARTICLE

Enzymatic processing of *Citrus reticulata* (Kinnow) pomace using naringinase and its valorization through preparation of nutritionally enriched pasta

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Abstract Citrus fruits are consumed either as whole fruits or as juice after processing. Processing of fruits yields a significant number of by-products in the form of pulp, peel and seeds, which are often discarded and major cause of environmental concern. Bitterness in the waste residue of citrus products is one of the leading hindrance in its valorization and supplementation in other food products. Aim of this study was to reduce the bitterness of *Citrus reticulata* (kinnow) pomace using enzymatic method and its supplementation in production of nutritionally rich pasta. Under optimized conditions (1U/mg enzyme naringinase concentration, temperature 50 °C, at pH 4.5 and treatment time 4 h), the maximum reduction (65.95%) of naringin (bitterness causing compound) was observed coupled with increase (60.13%) in naringenin (non-bitter compound). The debittered kinnow pomace has been further characterized for physio-chemical changes and morphological changes before and after treatment. The debittered kinnow pomace was then supplemented for the preparation of antioxidant and nutrient enriched pasta.

Keywords Kinnow pomace · Bitterness · Naringinase · Naringin · Naringenin · Pasta

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Introduction

Fruit processing industry plays a major role in agro-industrial sector. Brazil, China, India, Mexico, Spain and USA are major citrus producing countries worldwide (Satari and Karimi 2018). Citrus fruits consist of peels (40–55%), internal tissues (30–35%) and seeds (< 10%). In addition, citrus fruits are potent source of soluble dietary fibers along with various bio-active compounds such as flavonoids along with antioxidant properties (Lario et al. 2004). Processing of citrus fruits in industries generates large amount of peel waste (19 Mt annually) which can be used as raw material in bio-refinery (Bustamante et al. 2016). Peel and pulp are abundant source of sugars, minerals, dietary fibers and phenolics which have antioxidants, anti-mutagenic, cardio-preventive antibacterial activities.

"Kinnow," a hybrid between a king and willow mandarins (*Citrus nobilis Lour* × *C. deliciosa Tenora*) is one of the major citrus fruit crops in northern region of India (Sharma et al. 2007). India is the 5th largest producer of citrus fruits worldwide including kinnow mandarins, oranges, tangerines, lime and lemons. Orange group production including kinnow mandarins in India was about 4.75 million tonnes over 0.43 million hectares' area (Mahawar et al. 2019). Kinnow is the second most important fruit after grapes and cultivated in almost 125 countries of the world with 1155 million tonne production (FAOSTAT 2012; Nasir et al. 2016). Kinnow fruit is cherished fresh and peak harvesting seasons varies from November to February among the different part of India (Rafiq et al. 2018). Generally, kinnow juices and by-products have a

poor shelf life and mainly have postharvest losses (Panesar et al. 2009).

With increasing demand and consumption of kinnow fruit, large quantities of waste are also increasing. This waste is often an economic liability to the fruit processors, as waste disposal is a growing problem. Since kinnow processing residues does not find any significant commercial application in India, so the dumping and improper disposal of these by-products has created various disposal and environmental problems due to high sugar content and moisture, besides the loss of nutrients as these peels were rich source of cellulose, pectin, hemicelluloses, lignin, essential oils and phenolic compounds (Hou et al. 2013; Mejia et al. 2019). In addition, bitterness in the waste residue of citrus products is one of the leading hindrance in its valorization and causes of consumer unacceptability. Debittering of juices by using enzymatic hydrolysis can be a promising technique, however but reports on its application on kinnow pomace are not available. The citrus fibres were associated with naringin which is majorly responsible for bitterness in the kinnow industry by-products. The bitterness in citrus juices is derived from components such as predominantly naringin and their precursors. Naringin, possess flavanone-7-O-neo-hesperidoside structure, responsible for a bitter taste. The use of enzymatic methods for removal of bitterness causing compounds has been increased at a large pace and it helps in minimizing pollution problems (Puri and Banerjee 2000).

Although work has been reported on the debittering of citrus juices, however the data on the debittering of kinnow industry by-products is meagre. In view of the above, the present study has been focused on developing an enzymatic process to remove the bitterness from kinnow pomace residues, it characterization and valorization through preparation of nutritionally and antioxidant rich food product (pasta).

Materials and methods

Raw material

Kinnow juice industry waste containing pomace (peel, seeds and sac) left after juice extraction has been procured from *Punjab Agro Juices Limited,* Near *Abohar*, District Ferozepur (Punjab) India. Enzyme naringinase (NATE-0653, 50000U/g), Native *Thermomicrobia* sp. Naringinase (Rhamnosidase A) has been procured from Creative enzymes, U.S.A.

Chemical composition of kinnow pomace

Different nutritional properties and bioactive compounds present in kinnow pomace have been characterised by different methods such as moisture (using moisture analyzer, MA35, Saritorius), Protein (Lowry et al. 1951), Fat content (Rosenthal et al. 1996). Total phenols, (UPLC system, Water, USA), Dietary fibre (Fiber extractor, Velp, USA), DPPH activity (Shimada et al. 1992), total carbohydrates and ash, has been analyzed by using standard methods (AOAC 2000).

Enzymatic treatment of kinnow pomace

In this method, sample to water ratio (1:10) was maintained and seeds have been removed by centrifugation. The sample: solvent has been treated at (25–65 °C) for 2–6 h with the addition of (0–2.5%) enzyme naringinase NATE-0653 (Native *Thermomicrobia* sp.) at pH 4.5 (natural pH of kinnow pomace). Extracts were then passed through Whatman filter paper no.1 and centrifuged (Eppendorf Centrifuge 5810 R) at 8000 rpm for 3 min. The solid residue obtained was dried in hot air oven and powdered for further use.

Quantitative analysis of naringin and naringenin

Quantitative identification of naringin (bitterness causing compound) and non-bitter compound naringenin (produced after enzymatic reaction) has been determined by method used by Seal (2016) with slight modifications. Enzyme treated pomace samples were injected into Waters Acquity UPLC H-Class (Model: UPLC-H) (Milford, MA, USA) after filtration by 0.2-µm PTFE filter. UPLC consists of injector with Eclipse RP C18 column and Waters 2998 PDA (Photodiode Array) detector. Mixture of Solvent A-0.1% orthophosphoric acid in water (v/v) and Solvent-B acetonitrile has been used as Mobile phase. Column temperature was maintained at 30 °C and detector wavelength was set between 200 and 400 nm. 1 ml/min flow rate was induced having 254 nm UV detector for phenolic acids. Retention times and area of peaks have been compared with polyphenol standards and compounds were identified (mg/g). The analyses were done in triplicates.

Scanning electron microscopic analysis

Scanning Electron microscope (SEM) has been used to study morphological structure of food particles by creating three-dimensional images (Sharma and Bhardwaj 2019). SEM used in study was (SEM JCM-6000, JEOL Ltd, Tokyo, Japan). After pre-treatment and dehydration of sample, the sample having particle diameter < 100 µm has

been mounted after gold sputtering. Magnification has been done at different resolutions (500–2000 μm) to study the changes in microscopic structures and particles.

Fourier-transform infrared spectroscopic analysis

Fourier-Transform Infrared Spectroscopy (FTIR) with Attenuated total reflectance (ATR) (Agilent; Model: Cary 660 Series) has been used for the sample analysis. The range of 4000-600 cm^{-1} with a resolution of 4 cm^{-1} has been set (Grewal et al. 2017; Markoska et al. 2019) to evaluate the change in conformation. A Sample background spectrum has been scanned by using blank diamond ATR cell. Spectra was taken within region 3600–3200 cm^{-1} (–OH), 2926–2929 cm^{-1}(–CH), 1500–1700 cm^{-1} (COO–), 1300–1400 cm^{-1}(–CH_2), 1000–1022 cm^{-1}(CH–O–CH stretching). Data spectrum was normalized for the concentration by dividing the absorbance intensity at 1650 cm^{-1}.

Preparation of kinnow pomace enriched pasta

The enzyme treated oven-dried kinnow pomace has been incorporated at different concentrations (10–20%) in the preparation of pasta. In this method, semi-solid dough has been formed with mixture of semolina and treated kinnow pomace by addition of 30 ml water in single-screw pasta making machine (Dolly La Monferrina, Italy). Particular shape has been given to this pasta with the help of die in pasta making machine. The product formed was cut into desired length with the help of knife or cutter and placed in hot air oven at 60 °C to reduce the moisture content to 5–6%.

Organoleptic evaluation

Kinnow pomace samples after enzymatic treatment and the pasta after cooking were examined for organoleptic evaluation by group of panellists (n = 10, 7 males and 3 females, 23–36 years old) including Scientists and research students from Department of Food Engineering and Nutrition at Center of Innovative and Applied Bioprocessing, Mohali (India) at 30 ± 2 °C. Organoleptic evaluation has been made using nine point hedonic sensory score on the basis of appearance, taste color, aroma, body/texture, and flavour (Amerine et al. 1965) and the mean score of these all attributes was used to draw the overall acceptability. In pasta, the sensory evaluation has been made after cooking each sample. Before testing each sample panellists rinse the mouth with filtered water.

Statistical analysis

The data so obtained was analysed using Minitab Statistical software (Version 14.12.0, Minitab, State College, Pa., U.S.A). Results are expressed as mean ± SD. The difference between the mean values was tested for statistical significance (at 5% i.e. $p < 0.05$) using ANOVA followed Bonferroni post hoc test.

Results and discussion

Enzymatic treatment of kinnow pomace

Kinnow pomace was treated with Naringinase enzyme (0.5U/mg) at 40 °C for 6 h. It has been observed from the results that with the addition of naringinase enzyme, the hydrolysis of naringin and formation of naringenin got increased. After 4 h maximum reduction in naringin was 23.67% (from 2.201 to 1.680 mg/g) and increase in naringenin was 20.26% (from 0.301 to 0.362 mg/g).

Naringinase is an enzyme complex comprising of α-L-rhamnosidase and β-D glucosidase. This enzyme catalyzes the hydrolysis of naringin into prunine and rhamnose (Prakash et al. 2002). Prunin is further hydrolysed into tasteless naringenin (4,5,7 trihydroxy flavanone) and glucose. Activity of naringinase enzyme depends upon treatment time, temperature and enzyme concentration. So, optimization of these parameters has been carried out for maximum debittering of kinnow pomace.

Optimization of process parameters

Effect of enzyme concentration

Enzyme concentration has been varied from 0 to 2.5U/mg for 4 h at 40 °C to study the reduction of naringin and production of naringenin (Fig. 1). From the results it has

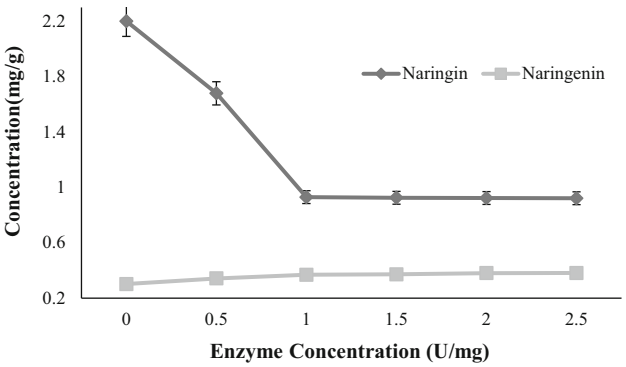


Fig. 1 Effect of naringinase concentration on the hydrolysis of naringin in kinnow pomace

been observed that maximum reduction in naringin 57.79% (from 2.201 to 0.929%) whereas, the formation of naringenin was 22.25% (from 0.301 to 0.368%) at 1U/mg enzyme concentration. Further increase in enzyme concentration has no significant effect on the hydrolysis of naringin. However, from the previous studies, it has been found that optimum activity of naringinase was at 1.0 g/L-0.25 g/L enzyme concentration at temperature of 40 °C after 4 h (Patil and Dhake 2014). From results as mentioned above, it has been found that maximum reduction has been achieved using lesser concentration of enzyme. So, from above results, for the maximum hydrolysis of naringin, 1U/mg enzyme concentration has been selected for further experimentation.

Effect of temperature

The effect of temperature (25–65 °C) on reduction of naringin and production of naringenin at 1U/mg enzyme concentration for 4 h has been shown in Fig. 2. From the results, it has been depicted that with an increase in temperature from 25 °C to 50 °C, there was increase in the naringin concentration; however, with further increase in temperature no subsequent increase in naringin reduction was observed. The maximum reduction in naringin to 65.87% (from 2.201 to 0.751 mg/g) and 59.80% (from 0.301 to 0.481 mg/g) has been observed at 50 °C in pomace. Thereafter, with increase in temperature no significant decrease in naringin has been observed. From the previous studies it has been found that optimum activity of naringinase enzyme varied from 40 °C to 50 °C at 1.0 g/L-0.25 g/L enzyme concentration (Radhakrishnan et al. 2013). It has also been observed that after 65 °C, the activity of naringinase keeps on declining with increase in temperature (Srikantha et al. 2017). Temperature is an important factor that has a profound effect on the activity of the enzymes. Enzyme show maximum activity at their optimum temperature and any change of temperature on either side of the optimum range can affect the performance of the enzyme.

Since maximum reduction of naringin was observed at 50 °C temperature, so it was selected in the further experimentation.

Effect of time

The effect of treatment time (1–6 h) on the decrease in the concentration of naringin with consistent increase in the concentration of naringenin has been shown in Fig. 3. Decrease in naringin concentration has been found to be 65.95% (2.20–0.75 mg/g) and increase in naringenin has been found to be 60.13% (0.30–0.48 mg/g). But after 4 h no significant reduction in concentration of naringin and increase in naringenin content have been observed. It has also been further reported in previous studies that with an increase in incubation time, the concentration of naringin keeps on decreasing in citrus fruit juice (Patil and Dhake 2014). It has also been reported that reduction in naringin content was not very significant from 1 to 3 h but after 3 h significant reduction in naringin has been reported (De-Silva et al., 2017).

From the above results, it has been concluded that optimum time for maximum reduction in naringin and maximum increase in naringenin was found at 1U/mg enzyme concentration, at 50 °C temperature and treatment time of 4 h.

On the basis of hedonic point scale, it has been found by the panellists that no bitter taste (overall acceptability: 8 ± 0.58) has been observed in enzyme treated kinnow pomace under optimized conditions (i.e. 1U/mg enzyme concentration at 50 °C for 4 h). Results from the linear optimization, sensory evaluation and quantification studies by HPLC, it has been concluded that treatment of kinnow pomace with 1U/mg of enzyme concentration for 4 h at 50 °C resulted in removal of bitterness to the level of acceptance.

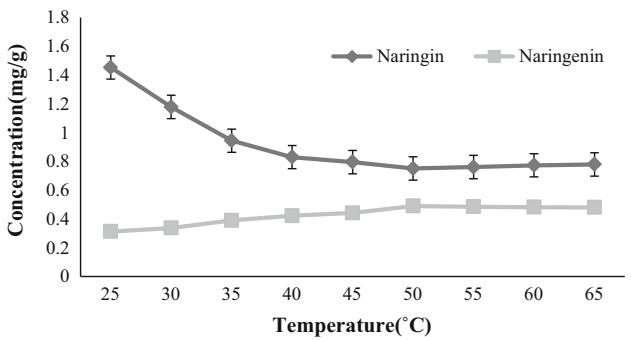


Fig. 2 Effect of temperature on the hydrolysis of naringin in kinnow pomace using enzyme naringinase

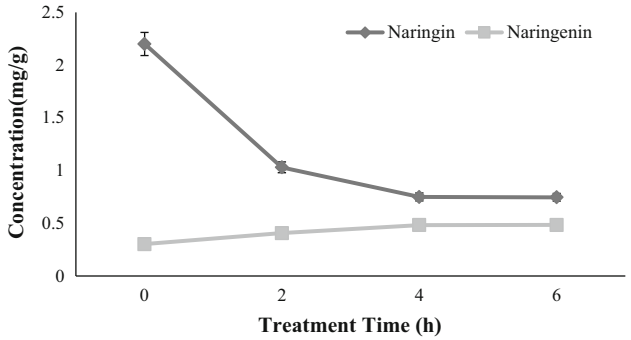


Fig. 3 Effect of treatment time on the hydrolysis of naringin in kinnow pomace using enzyme naringinase

Chemical composition of kinnow pomace before and after enzymatic treatment

Chemical composition of kinnow pomace before and after enzymatic treatment has been shown in Table 1. In untreated kinnow pomace, total carbohydrate observed was 74.35 mg/g and ash content in kinnow pomace was found to be 0.61% (Singla et al. 2019). The pectin content was 5.42% these results were in accordance to the previous reports (Marin et al. 2007). Total phenolic content was found to be 1.98 mg/g, Dietary fibre and DPPH activity has been found to be 60.41% and 68.02% kinnow pomace, respectively.

It has been observed that after naringinase treatment, DPPH activity of kinnow pomace got increased to 81.70%. Enzymatic treatment in grape pomace and grape seeds resulted in generation of free phenolic compounds that can result in either directly liberation of antioxidant phenols or conversion into more potent antioxidant compounds (Chamorro et al. 2012). It has also been reported that the antioxidant activity of concentrated orange juice after enzymatic biotransformation of naringin to naringenin has increased to 70%. From in-vitro-studies it has been observed that it helped in increasing the bioactivity and functionality (Ferreria et al. 2013). Enzymatic treatment resulted in formation of simple glucose and rhamnose sugars, as complex sugars got broke down into simple sugars. Dietary fibre content also got increased (64.03%) which is directly related to increase in soluble fibre content.

Scanning electron microscopic studies

Scanning electron microscopy (SEM) technique is used to study the surface morphology. SEM images of kinnow pomace before and after enzymatic treatment have been shown in Fig. 4 (a, b). Significant morphological changes in terms of clear fiber have been visualized in kinnow pomace after treatment with respect to untreated pomace. After enzymatic treatment, massive swelling may have taken place that break down the cells easily which leads to release of phenolic compounds (Mushtaq et al. 2014).

Fourier-transform infrared spectroscopic studies

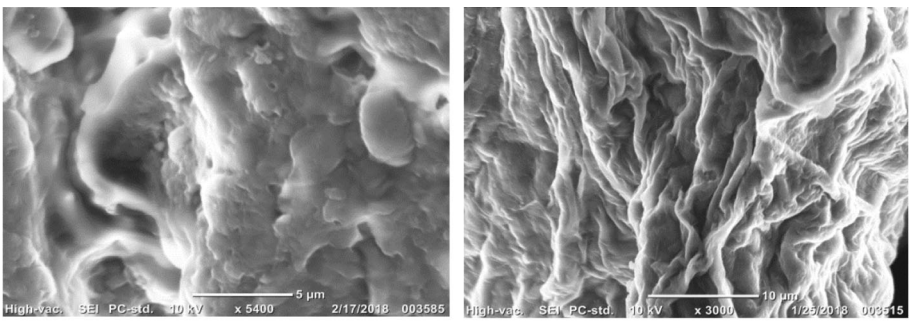
Fourier-Transform Infrared (FTIR) spectroscopy was used to examine the structural changes on the fiber surface before and after enzymatic treatment. FTIR images of cellulose standard, pectin and enzyme treated kinnow pomace have been shown in Fig. 5. Absorption wavelength of enzyme treated kinnow pomace has been compared with FTIR spectra of standards such as cellulose and pectin to confirm the effectiveness of enzymatic treatment. The absorption wavelengths of pure compounds of cellulose, pectin, and enzyme treated kinnow pomace were found

Table 1 Chemical composition of kinnow pomace before and after naringinase enzyme treatment

Parameters	Kinnow pomace	Enzyme treated kinnow pomace
Ash (%)	0.61 ± 0.05^{a}	0.7 ± 1.03^{b}
Fat (%)	1.61 ± 0.71^{a}	0.41 ± 0.03^{b}
Dietary fibre (%)	45.11 ± 0.04^{a}	64.03 ± 0.04^{b}
Total Phenols (mg/g)	1.98 ± 0.71^{a}	1.63 ± 0.04^{b}
Pectin (%)	5.42 ± 1.26^{a}	2.09 ± 0.09^{b}
DPPH activity (%)	68.02 ± 1.65^{a}	81.7 ± 1.72^{b}

Mean ± SD ($N = 3$)

Different superscripts in the same rows indicate the significant difference ($p < 0.05$)



a Untreated pomace **b** Enzyme treated pomace

Fig. 4 SEM images of kinnow pomace before and after naringinase enzyme treatment **a** Untreated kinnow pomace, **b** Enzyme treated pomace

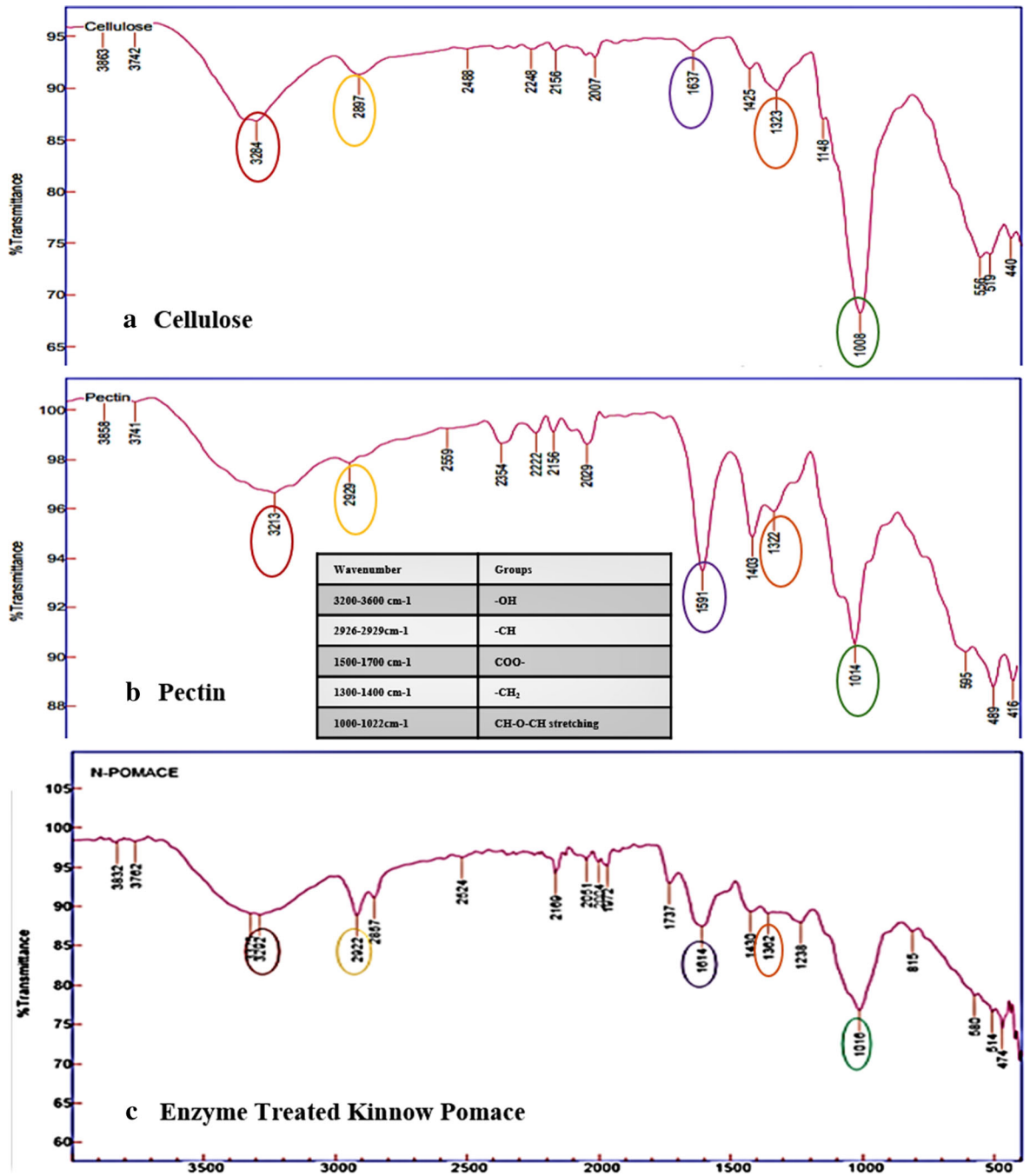


Fig. 5 FTIR of enzyme treated kinnow pomace **a** Cellulose, **b** Pectin, **c** Enzyme treated kinnow pomace

within the range of 1500–1700 cm^{-1} which was attributed to the presence of COO– group. Also, wave numbers 3200–3600 cm^{-1}attributed to –OH group, 2926–2929 cm^{-1} to –CH group, 1300–1400 cm^{-1} to $–CH_2$ group and 1000–1022 $cm^{-1}$to CH–O–CH stretching, respectively. Increase in free radical activity results in the increase in the number of free hydroxyl groups in reaction medium, due to increase in hydrogen donation to free radicals (Jung et al. 2006; Chamorro et al. 2012).

Utilization of enzyme treated kinnow pomace in the preparation of pasta

Debittered kinnow pomace after enzymatic treatment has been incorporated in the preparation of pasta. As the kinnow pomace is rich in fibers, phyto-chemicals and antioxidants, so the supplementation of debittered kinnow pomace in the pasta resulted in the production of nutrient and antioxidant rich pasta. From the results of hedonic

scale reading, panellists observed the original flavor, having appealing orange colour, body/texture of pasta which is highly acceptable (overall acceptability: 8 ± 0.21). The studies have indicated that the enzyme processed kinnow pomace can be successfully used for the preparation of nutritionally rich pasta and other extruded food products.

Conclusion

Effective reduction in bitterness caused by naringin in kinnow pomace has been observed by the treatment of enzyme naringinase (NATE-0653) in kinnow juice industry processing waste (pomace). Under optimal conditions (1U/mg of enzyme concentration at 50 °C for 4 h), 65.95% reduction of naringin and 60.13% increase in non-bitter naringenin has been obtained after treatment of kinnow pomace with naringinase enzyme, which resulted in reduction of bitterness to threshold limit of acceptance. This enzyme treated debittered kinnow pomace has been successfully incorporated into pasta to increase the nutritional benefits of extruded food products. The developed process can result in dual benefits, i.e. minimization of the pollution problems caused by the kinnow processing by-products along with production of nutritionally rich extruded food products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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