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Cellulolytic Treatment: A Competent Approach to Improve Extraction and Storage Stability of Carotenoids from Kinnow (*Citrus reticulate*) Peel

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The purified cellulolytic enzyme was applied in carotenoid extraction from the Kinnow mandarin peel. The results revealed that the highest yield (8.60 ± 0.44 mg/100 g peel) of carotenoids was observed in T_4, a combination of 250 IU both of CMCase and Pectinase/100 g peel whereas, the minimum recovery (3.21 ± 0.17 mg/100 g peel) was found in case of T_0 (control). To assess the storage stability, the extracted carotenoids were subjected to different conditions for light and temperature. When the pigment was stored at 30 °C in dark, it showed more stability than in light. Similarly, pigment stability was adversely affected by increasing storage temperature and the loss was more pronounced at higher temperature as compared to refrigeration. After freeze drying, the stability of the extracted pigment was assessed; high retention was observed when stored in darkness or under refrigerated conditions.

Keywords: Cellulase, Carotenoids, Kinnow, Enzymatic extraction.

INTRODUCTION

Kinnow mandarin (*Citrus reticulata*) is the popular form of citrus fruit in Pakistan, because of its unique taste and flavour as well as the heat tolerance character inherited from its parent cultivar King. The core production area of Kinnow lies in central Punjab of Pakistan [1]. The cultivar has attractive fruit colour, size and good eating quality, deep yellowish orange flesh colour, juicy and rich, aromatic and distinctive flavour. Kinnow fruit production is mostly intended for fresh fruit market and only a few processors go for its juice extraction. It has assumed special economic importance and export demand due to its high juice content, unique flavour & taste as well as a rich source of vitamin-C. Additionally, it also provides vitamin A, B_1, B_6, calcium, folic acid, iron, magnesium and potassium. Besides, it is rich in β-carotene and bioflavonoid which are essential elements for a healthy life [2,3]. Although the juice yield of citrus like Kinnow is practically less than fruit weight, massive amounts of byproducts including peel and seeds are produced every year [4,5]. Such byproducts/wastes are traditionally used as molasses for animal feed [6], pectin extraction [7] and fuel production [8]. Recent studies have explored some fruits or vegetable byproducts as source of natural antioxidants and other valuable products [8,9]. Among these compounds, carotenoids are most important which also act as natural colouring pigments.

Interest in the production of natural pigments is growing due to limitations on different certified food colours. There is a continuous demand for natural pigments, like carotenoids, as food colourants because of their desirable properties such as natural origin, null toxicity and high adaptability [10,11]. The covalent bonding between carotenoids and proteins prevents the pigment oxidation. However, after solvent extraction, the pigments get separated from the proteins, become water insoluble and prone to oxidation. The enzymatic extraction process is a good alternate to handle this problem. Enzymatic preparations including CMCase disintegrate the plant tissues to improve the pigment extraction yields [12]. The hydrolytic enzymes interact on cell walls, breaking down the structural integrity rendering the intracellular materials more exposed for extraction. As these pigments remain in their natural state still bound with proteins, these are more stable as compared to those obtained through traditional methods [13,14]. Therefore, the present study was aimed to get valuable product from the Kinnow peel which is otherwise underutilized in Pakistan and other developing countries.

EXPERIMENTAL

Kinnow peel obtained from the local industry was used for the extraction of carotenoid pigments. The CMCase produced at National Institute of Food Science and Technology,

University of Agriculture Faisalabad was used singly and in combination with pectinase. Celites Filter Cel was purchased from Fluka Chemical Corp., Ronkonkoma, NY, USA.

Sample preparation: The albedo of kinnow peel was abraded off using peeling knife. Rest of the peel *i.e*. flavedo was chopped and homogenized in deionized water by warring blender to reduce the particle size and increase the surface area for enzyme action.

Enzymatic treatment: In 100 g of ground and homogenized peel, CMCase and pectinase were added together with 200 mL of distilled water. The samples were stirred for 6, 12, 18 and 24 h at room temperature. The enzymes were used in combinations given in (Table-1).

TABLE-1
LEVELS OF THE ENZYMES USED IN THE CAROTENOID EXTRACTION

Treatments	CMCase (IU/100 g peel)	Pectinase (IU/100 g peel)
T_0	0	–
T_1	250	–
T_2	500	–
T_3	125	125
T_4	250	250

Extraction of carotenoids: After enzyme treatment, the mixture was vacuum filtered, first for water soluble pigment extract and then the residue was washed with 95 mL/100 mL ethanol. The ethanol then was evaporated by using rotary evaporator. The remaining residue was carotenoid pigment and water.

Measurement of carotenoid: Carotenoid concentration in duplicate samples was estimated by UV-visible spectrophotometer (CECIL 7200) at 450 nm. Absorbance values of samples were converted to percentage retention for accurate comparison among the treatments.

Pigment yield: Carotenoid yield was calculated as a result of enzymatic treatment of kinnow peel using the following expression.

$$\mu g \text{ carotenoid/g sample} = (A_{450})\ (D)\ (105/181)\ (\text{light path (cm)})\ (\text{sample weight g})$$

wherein: A_{450} = The absorbance of the solution at 450 nm; D = The dilution factor based on 100 mL of solvent solution

Stability tests: For the stability tests, the effects of light, dark and temperature on carotenoids was investigated [15].

Statistical analysis: Complete randomized design (CRD) was applied and to determine the level of significance, the data obtained for each parameter was subjected to analysis of variance (ANOVA) technique as described by Steel and Torrie [16]. Duncan's multiple range test (DMR) was used to compare the means at 5 % level of probability using CoStat Statistical Software (2003).

RESULTS AND DISCUSSION

Carotenoids are responsible for many of the red, orange and yellow colours of fruits and vegetables. Traditionally, solvent extraction is used for the carotenoid production [14,17]. Being highly unsaturated molecules, the pigments are prone to isomerization, which causes colour loss and oxidation. However, in enzyme extraction the enzymes disrupt the cell wall and release the carotenoids in the chloroplasts and in cell fluids. The carotenoids are still bound to proteins and hence keep their natural state which provides stability to the highly unsaturated pigment structure and the colour remains more stable during the storage.

Extraction yield of carotenoids from Kinnow Mandarin peel: The purified CMCase along with pectinase was utilized in the extraction of carotenoids from the Kinnow peel. The mean squares in Table-2 elucidated that treatments, incubation time and their interaction exhibited significant effect on the pigment yield from the kinnow peel. The mean values for the pigment yield (dry weight basis) indicated that the maximum yield (8.60 ± 0.44 mg/100 g peel) of carotenoids was observed in T_4 followed by T_3 (7.03 ± 1.51 mg/100 g peel) whereas, the minimum recovery (3.21 ± 0.17 mg/100 g peel) was calculated in case of T_0 (Table-3).

TABLE-2
MEAN SQUARES FOR CAROTENOID YIELD THROUGH ENZYMATIC EXTRACTION

SOV	df	Carotenoid extraction
Treatments	4	51.630**
Incubation Time	3	60.506**
Treatments × Incubation time	12	1.391**
Error	40	0.077
Total	59	

** Highly significant

The results regarding incubation time explicated that in all cases, minimum yield was observed at the initiation of the trial whereas; maximum was recorded at 24 h of incubation. The time reflected a positive correlation with that of pigment yield and carotenoid recovery continued to increase with the passage of time in all the cases. However in case of T_3 and T_4, some consistency was found in the yield after 18 h and thereafter, slight increase in carotenoid yield was observed at 24 h. Increase in enzyme concentration and combination of both enzymes showed linear correlation with pigment recovery. Enhanced extraction yield for lutein after treatment of the dried and powdered flower petals with enzymes has also been

TABLE-3
CAROTENOID YIELD AFTER ENZYMATIC EXTRACTION (mg/100 g PEEL)

Treatments	Incubation time (h)				Means	CMCase	Pectinase
	6	12	18	24			
T_0	1.55	2.15	3.43	5.71	3.21 ± 0.17 e	-	-
T_1	2.62	3.83	6.11	6.85	4.85 ± 0.21 d	250 IU/100 g	-
T_2	2.74	4.13	6.79	7.64	5.33 ± 0.34 c	500 IU/100 g	-
T_3	3.82	6.41	8.76	9.15	7.03 ± 0.51 b	125 IU/100 g	125 IU/100 g
T_4	5.84	9.06	9.75	9.76	8.60 ± 0.44 a	250 IU/100 g	250 IU/100 g

reported by Tekwani and D'mello [9] and Lazore *et al.* [18] for raspberry.

The enzymes caused increase in the recovery through break down of the cell wall material and released the carotenoids in the chloroplasts and cell fluids. Because cellulose in the plant materials is covered by pectin therefore, T_0 and the treatments employing CMCase alone exhibited increase in yield after prolonged incubation time. Since, the two enzymes reflect synergism in the action consequently; T_3 and T_4 resulted in faster release of the pigments. The increase in the yield was more pronounced during the initial hour of trial whereas; less increase in the yield was noted after extended periods. The synergistic effect of the two enzymes has also been reported by earlier researchers [13]. Similar results have been reported by Cinar [19] that confirms the synergism between the two enzymes (cellulases and pectinases).

Stability studies: To assess the storage stability, the carotenoids extracted from kinnow peel were subjected to different conditions for light and temperature.

Effect of temperature and light: The means depicted in Fig. 1 elucidated the effect of storage conditions including light and temperature on the stability of solvent extracted carotenoids. The results expounded that light as well as temperature exerted significant effects on the stability of carotenoids and there was an inverse correlation between the pigment stability and storage time.

When the carotenoids were stored at 30 °C in light; after 10 days it lost 50.5 % of its activity whereas, storage for same duration at same temperature under darkness exhibited loss of 25.2 %. After 50 days of pigment storage, 88.3 % pigment loss was recorded in samples stored at 30 °C in light while 64.4 % loss was noted in samples stored under darkness at same temperature. Likewise, more pigment loss was observed at 45 °C (92.8 %). Storage of pigment samples at 4 °C resulted in minimum loss (35.4 %) during the entire duration of study.

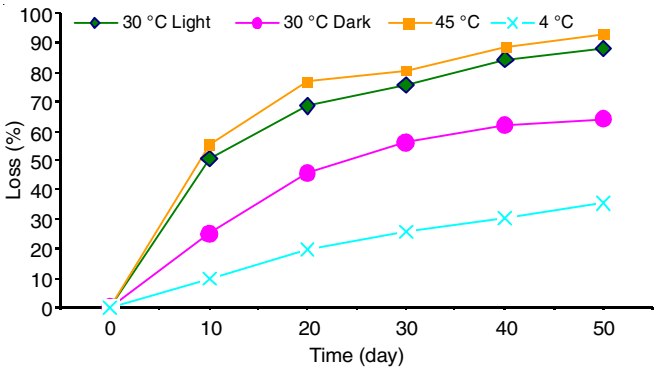

Fig. 1. Effect of light and temperature on the loss of solvent extracted carotenoids

Fig. 2 depicts storage stability of enzyme extracted carotenoids at various storage conditions. The exposure of carotenoids to light resulted in greater losses (72.4 %) than that of darkness (64.3 %) at 30 ºC during the entire duration. Likewise, it is obvious from Fig. 2 that there was an inverse correlation between rise in storage temperature and the pigment stability; the loss at elevated temperature (45 ºC) was 2.8 fold higher than that of refrigerated conditions (4 ºC).

The findings of present study explicated that the enzyme extracted pigments were more stable towards varied environmental conditions than the solvent extracted carotenoids. The losses were more pronounced in solvent extracted pigments as compared to enzyme aided extracted pigments. The results are also supported by those of Cinar [15,19] who reported the enhanced stability of the enzyme extracted plant pigments at various temperatures and storage conditions. Since the enzyme extracted pigments are bound to the proteins and remain in their native state therefore, exhibit more stability towards adverse conditions of the environment that supports the present results.

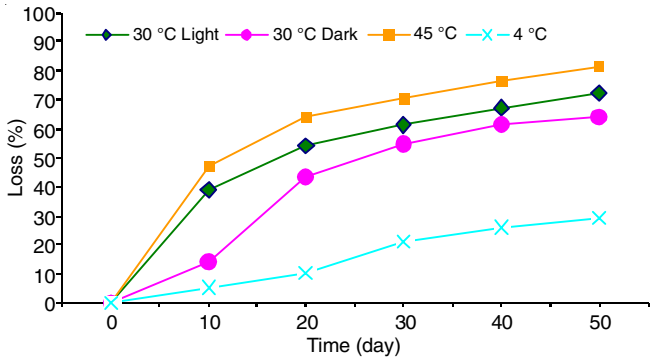

Fig. 2. Effect of light and temperature on the loss of enzyme extracted carotenoids

Effect of freeze drying: The means showing the effects of different storage conditions over time on stability of freeze dried solvent extracted pigments indicated negative correlation (Fig. 3) between pigment stability and storage. The mean values indicated that after 10 days there was 25.4 % loss in the pigment stored in light at 30 °C whereas, 23.1 % loss was observed when the pigment was stored in darkness at the same temperature. At the end of study *i.e.* 50 days the loss in the carotenoids reached 49.9 and 40.3 % in light and darkness, respectively. The means explicated that storage of carotenoids at high temperature resulted in augmented pigment loss. At the 10^{th} day of storage, 5.3 % loss of pigment was calculated at 4 °C, while in samples stored at 45 °C the loss reached up to 27.2 %. Likewise, after 50 days, 62.1 % of the pigment was lost at 45 °C as compared to 19.2 % pigment loss in samples stored at 4 °C.

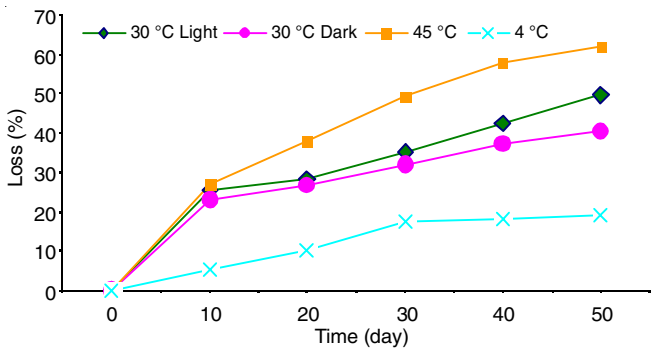

Fig. 3. Effect of light and temperature on the loss of freeze dried solvent extracted carotenoids

While comparing the effect of light and darkness during storage (Fig. 4) it was found that exposure of freeze dried

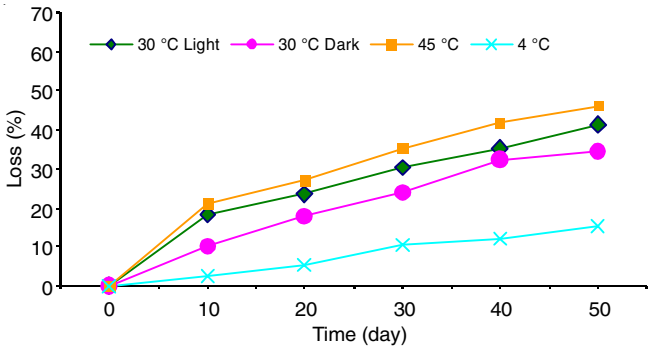

Fig. 4. Effect of light and temperature on the loss of freeze dried enzymes extracted carotenoids

enzyme extracted carotenoids to light for extended periods *i.e.* 50 days resulted in greater losses (41.1 %) than darkness (34.3 %). In the same way, the elevated temperature had negative impact on the pigment stability; 46.1 % pigment loss at 45 °C whereas, during refrigeration only 15.2 % loss was recorded.

Tang and Chen [20] and Cinar [15,19] have reported the stability of enzyme extracted freeze-dried carotenoid pigments under different storage conditions. Enzyme extracted pigments remain bound with proteins through covalent bonding or weak interactions that prevents pigment oxidation [14]. The findings of this study are in line with the results found by Desobry *et al*. [21] who stated the freeze drying as the most effective way of preserving carotenoids from the carrots. Park [22] reported that the freeze drying significantly reduced carotene degradation of carrot during storage. The loss of the carotenoids increased at elevated rather than lower temperatures. Similar trend was found by Arya *et al*. [23] who observed 32.7 % loss in freeze dried papaya pigment at room temperature and 42.0 % at 37 °C after 45 days. Delia and Amaya [24] also noted a 53 % loss of carotenoid pigments in freeze dried squashes at room temperature after 3 months.

Conclusion

The results for the extraction and storage stability of the carotenoids indicated that enzymatic treatment of kinnow peel resulted in higher carotenoid yields. However, more pronounced effects were observed when combinations of both enzymes were employed during the process. The extracted pigment showed more stability when stored in dark under refrigerated conditions. Moreover, the freeze drying of t/he extracted pigment was found to be advantageous for increasing the storage stability.

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