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Research article

ENZYMATIC CAROTENOID CLEAVAGE IN YELLOW PASSION FRUIT (*Passiflora flavicarpa*) POMACE

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Abstract
Carotenoid Cleavage Dioxygenase (CCD) is a type of enzyme that plays a role in forming flavor compounds from natural carotenoid sources, such as yellow passion fruit. The study aims to describe the partial purification of CCD enzymes from yellow passion fruit pomace and to identify the optimum pH and temperature performance of CCD enzymes, carotenoid compounds, and volatile compounds in yellow passion fruit pomace. The enzyme was partially purified using centrifugation, 80% ammonium sulfate precipitation, dialysis with a membrane cutoff of 12 kDa, and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The relative activity of CCD enzymes on dialysis was 31.94%, showing that there was CCD enzyme activity that can oxidize the β-carotene substrate. The optimum temperature of CCD enzymes was 60 °C, while the optimum pH value was 7. The analysis of carotenoids using HPLC-PDA showed that yellow passion fruit pomace contains β-carotene, neoxanthin, ζ-carotene, and luteinepoxide. The dominant protein produced by dialysis had a molecular weight of 40 kDa. There were 19 volatile compounds identified in the yellow passion fruit pomace. The activity of CCD enzymes from yellow passion fruit pomace using carotenoid substrate can be used as a biocatalyst in the natural flavor industry

1. Introduction

Several exotic fruits are commonly used for juice-making in several countries. Unfortunately, after the juice production, the pomace as a by-product has not been utilized completely. The agricultural byproducts, which are widely available and rich in organic chemicals, offer both a challenge and an opportunity (Rame *et al*., 2023; Al-Baarri *et al.*, 2019). Yellow passion fruit is one of the fruits that are usually used for juice making and producing byproducts. Yellow passion fruit is

type of passion fruit that contains beneficial chemical compounds, such as citric acid, ascorbic acid, lactic acid, malonic acid, niacin, riboflavin, vitamin A and C (Ayuningtyas & Broto, 2023). The residues from passion fruit processing represent 76% of the weight of the processed fruits, of which 26% is made up of the seeds (Ferrari *et al*., 2004). Annually, these seeds amount to tons of solid residues whose disposal involves operational costs for the pulp and juice industries and represents an environmental problem (Malacrida & Jorge, 2012). Whereas by-products of exotic fruits still contain high levels of various health-enhancing substances that can be extracted from the byproducts, for example, as a raw material for nutraceutical, and nutritional, and can be used for enzyme production (Ayala-Zavala *et al*., 2011; Ruiz-Sola & Rodríguez-Concepción, 2012). The primary health benefits of carotenoids are ascribed to their antioxidant properties (Andarwulan *et al*., 2021).

Carotenoid cleavage dioxygenases (CCD) are a family of enzymes that catalyze the oxidative cleavage of carotenoids. The distinctive yellow, orange, and red colors of fruits and vegetables are caused by phytonutrients called carotenoids (Anjani *et al*., 2022). This CCD enzyme can be used as a precursor for natural flavor formation. In a previous study, thirteen carotenoids were identified and separated in passion fruit using HPLC, with z-carotene identified as the predominant compound (Mercadante *et al*., 1998). β-carotene and lycopene were also identified in passion fruit by-products (Da Silva *et al*., 2014). Yellow passion fruit contains β-ionon and other volatile compounds from the breakdown of non-volatile precursors (Janzantti *et al*., 2012; Leão *et al*., 2014). The presence of β-ionons and carotenoids indicates that in passion fruit, a CCD enzyme breaks down carotenoids into volatile compounds. The formation of or isoprenoids as aroma-impact compounds by enzymatic reaction has been identified in several plants, e.g., star-fruits, nectarines, quince, rose, osmanthus flower, seaweed, tomato, petunia flower, melon, citrus (Baldermann *et al*., 2005, 2010; Fleischmann, Lutz-Röder, *et al*., 2002; Fleischmann, Studer, *et al*., 2002; Huang *et al*., 2009; Simkin *et al*., 2004). CCD enzymes can degrade β-carotene to β-ionon which has a fruity-like aroma.

This study aimed to identify carotenoid compounds with HPLC-PDA, describe partial purification of CCD enzymes from yellow passion fruit pomace, identify the optimum pH, temperature performance, and molecular weight of CCD enzymes, and identify volatile compounds in yellow passion fruit pomace by using GC/MS.

2. Materials and methods

2.1 Materials

Yellow passion fruit *(Passiflora flavicarpa)* has been obtained from Lintang Panglipuran farm, Sleman, Yogyakarta, Indonesia. β-Carotene was purchased from Sigma-Aldrich, America. Tris–HCl, MgCl2, KCl, Tween 40, sodium dodecyl sulfate (SDS), acrylamide, and bisacrylamide were all purchased from Merck, Germany. All other reagents were at least of analytical grade.

2.2 Methods

2.2.1 Identification of carotenoid compounds

Carotenoids were isolated from pandan leaves using the method published by Ningrum *et al* (Ningrum *et al*., 2015), with some modifications. Three grams of the plant were powdered and extracted with 30 mL of hexane, acetone, and methanol (2:1:1, v/v/v), which was repeated until the extraction solvent was colorless. The extract was then saponified by adding 8 mL of 40% methanolic potassium hydroxide for 1 hour. Next, 30 mL of hexane was used to extract the carotenoids. The extract was then rinsed with distilled water until it reached neutral pH and dried with sodium sulphate. After evaporation, the residue was soaked in acetone and filtered through a 0.45 μm membrane filter for HPLC-PDA analysis.

A C18 reverse-phase analytical column was utilized with a gradient solvent system of acetone (A) and water (B). Initially, a mixture of 70% acetone and 30% water was used, with a linear increase to 95% acetone over 20 minutes, and then further increased to 100% acetone by

50 minutes. The flow rate was set to 0.5 mL/min, with wavelength scanning between 350 and 550 nm. The HPLC system (Accela, Thermo) featured a quaternary gradient and a photodiode array detector. Semi-quantitative analysis of other carotenoids was achieved by applying peak areas to a calibration curve based on β-carotene.

2.2.2. Enzyme purification

The pulp of fully ripened yellow passion fruit (1 kg) was processed to make juice. After filtration, the remaining pomace, a by-product of the juice extraction, was collected. The pomace was homogenized in a sample buffer (125 mmol/l KCl, 5 mmol/l $MgCl_2$, 50 mmol/l Tris, pH 7, 1:2 w/v) using a blender for 120 seconds. The homogenized mixture was centrifuged at 7000 g at 20 °C for 10 minutes, after which the sediment was discarded, and the supernatant underwent ammonium sulfate precipitation (80%, 24 hours, 4 °C). The resulting precipitate was dissolved in sample buffer and dialyzed using a membrane with a 12 kDa cutoff at 4 °C for 24 hours, with the buffer replaced three times. Protein concentration was determined using the Lowry assay, with bovine serum albumin as the standard.

2.2.3. Enzyme assay

The carotenoid-cleavage activity was measured following the method outlined in (Huang et al., 2009). The breakdown of the substrate, β-carotene, was analyzed using a UV/VIS spectrophotometer at 505 nm. In each enzymatic assay, a reaction mixture containing the enzyme extract, a carotenoid/Tween 40 substrate, and a buffer solution was incubated. The buffer solution consisted of 100 mM Tris-base, 125 mM KCl, and 5 mM $MgCl_2$. The carotenoid/Tween 40 substrate solution was prepared according to (Huang *et al*., 2009): 1 mg of β-carotene was dissolved in acetone, combined with 1 g of Tween 40 also dissolved in acetone, thoroughly mixed, and the solvent was evaporated. The remaining mixture was diluted with 10 mL of H_2O and filtered through a 0.45-mm filter.

Enzymatic activity was assessed by measuring the absorbance of each sample (250 mL of crude enzyme, 1675 mL of Tris buffer, and 75 mL of carotenoid substrate) and control (1925 mL of Tris buffer and 75 mL of carotenoid substrate) over 20 minutes at 505 nm, starting immediately after substrate addition. The substrate's degradation was monitored through time-course measurements for the entire 20 minutes. The change in absorbance before and after incubation was used to calculate relative enzymatic activity. Each experiment was conducted in triplicate.

2.2.4. Influence of temperature and pH on the enzymatic activity

The effect of temperature on carotenoid-cleavage activity was evaluated by incubating the enzymatic reaction mixture at several temperatures (30, 45, 60, 75, 90 °C) for 10 min. The effect of pH on carotenoid-cleavage activity was evaluated at several pH values of the Tris buffer containing 100 mm Tris-base, 125 mm KCl, and 5 mm $MgCl_2$ (pH 5-9). Then, the activity was spectrophotometrically determined, as described above. All experiments were performed in triplicate.

2.2.5. Determination of molecular weight

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini Protein Cell (BioRad) using commercially available 30% polyacrylamide gels stained with commercially available Coomassie blue. All runs were carried out under constant voltage conditions (100 V) at room temperature for ± 2 h.

2.2.6. Volatile compounds

Ten grams of yellow passion fruit pomace was a mixture with pentane:dichlorometan (2:1 v/v). The mixture was stored in the freezer for 24 h. After that, the solution was filtered through a 0.45 μm membrane filter. Filtrate was added sodium anhydrous and evaporated. The free volatile compounds were analysed by gas chromatography-mass spectrometry (GC-MS) using the following temperature program: initial temperature 80 °C, ramped to 250 °C at 10 °C/min and held for 10 min. The mass scan range was set to m/z 50–300. Helium was used as carrier gas at a constant flow rate of 1.7 mL/min. The identification of volatile compounds was based on a comparison of the mass spectra of each component with the mass spectral library WILEY.

3. Results and discussions

3.1 Identification carotenoid compounds

Yellow passion fruit has orange pulp, which indicates carotenoids. In this study, carotenoid compounds were identified to determine the types of carotenoids contained in yellow passion fruit pomace as CCD enzyme substrates. Carotenoids in yellow passion fruit pomace extracts were identified by comparison with retention time and absorption spectrum. In this study, the identification of carotenoid compounds was performed by comparing the chromatogram of the sample with that of the standard using an overlay method. This method enables the retention times of both the sample and the standard to align in the same positions. Peaks with retention times that correspond to the standard are identified as belonging to the sample. Furthermore, carotenoid identification can also be achieved by comparing the spectrum generated by the standard with that of the sample; both spectra should correspond closely.

In Figure 1, there are four identified carotenoids, including neoxanthin, z-carotene, b-carotene, and luteinepoxide. The chromatogram shows the presence of four identified carotenoid peaks: at a retention time of 26 minutes is ζ-carotene, at 27 minutes is neoxanthin, at 28 minutes is β-carotene, and from 29 to 30 minutes is luteinepoxide In the previous studies, it was found that yellow passion fruit found thirteen types of carotenoids identified; phytoene, phytofluene, β-carotene, ζ-carotene, neurosporene, lycopene, prolicopene, monoepoxy β-carotene, β-cryptoxanthin, β-citraurin, antheraxanthin, violaxanthin and neoxanthin (Mercadante *et al*., 1998). Another study reported on yellow passion fruit containing carotenoid compounds such as; lutein, luteinepoxide, lycopene, zeaxanthin, β-carotene and β-cryptoxanthin as the dominant carotenoid compounds (Pertuzatti *et al*., 2015). The existence of differences in carotenoid compounds can be due to several factors, such as different varieties, different extraction methods, and the condition of the equipment used.

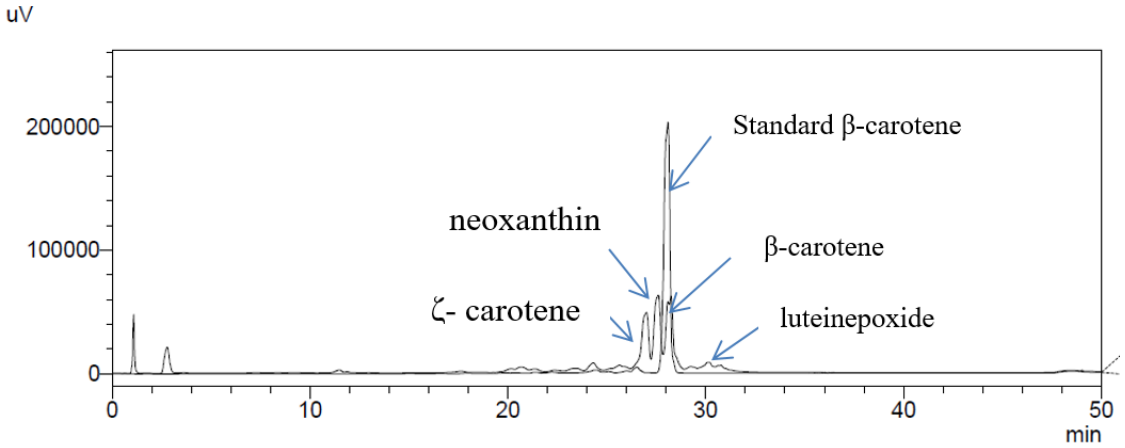


Figure 1. HPLC chromatogram of carotenoids of yellow passion fruit pomace

Table 1 shows information regarding retention time, absorption maxima, and semi-quantification of the carotenoids found in yellow passion fruit pomace. β-Carotene, neoxanthin, and lutein were found as major carotenoids in HPLC chromatogram (Figure 1). The major carotenoids content of yellow passion fruit pomace consisted of 9,51 ppm of neoxanthin, 9,41 ppm of β-carotene and 7,44 of ζ- carotene.

Table 1. Carotenoids identified in yellow passion fruit pomace by using HPLC analysis

Carotenoid	Peak	Retention time (min)	λ_{max} (nm)	Literatur (Britton *et al*., 1995)	Semi quantification (ppm)
ζ- carotene	1		380	380	7.44
	2	26.998	401	402	
	3		426	426	
Neoxanthin	1		412	416	9.51
	2	27.597	435	438	
	3		464	466	
β - carotene	1		427	-	9.41
	2	28.087	450	454	
	3		478	480	
Luteinepoxide	1		419	416	0.93
	2	29.238	441	440	
	3		467	468	

Table 1 shows several variations in the maximum wavelengths formed and indicates a shift in wavelength between the sample and the literature. This can be caused by several factors, including interactions with types of carotenoids present in the extract, the acidity level of the solution, oxidation effects, and other factors (Britton *et al*., 1995). Once the types of carotenoids in the sample are identified, the carotenoid content can be calculated semi-quantitatively by applying the obtained peak height to the β-carotene calibration. The results of this study show that carotenoids are still present in the yellow passion fruit pomace. This was also found in the study by Da Silva *et al*. (2014), which reported that by-products from several tropical fruits, such as pineapple, acerola, guava, and passion fruit in Brazil, contain higher levels of β-carotene, lycopene, flavonoids, and anthocyanins compared to their juice. In addition to serving as precursors for carotenoid-derived aroma compounds, the presence of carotenoids in yellow passion fruit pomace and juice indicates that carotenoids act as provitamin A, which plays an important role in eye health and have antioxidant activity (Murillo *et al*., 2013).

3.2.Isolation and characterization of yellow passion fruit pomace carotenoid cleavage enzyme

As can be seen in **Table 2**, each stage of CCD enzyme purification from yellow passion fruit pomace was increased. Increased activity of CCD enzymes after precipitation of ammonium sulfate and dialysis compared with crude enzymes. The relative activity of crude enzyme from yellow passion fruit pomace was 20.30 ± 0.65% which increased after precipitation to 27.28 ± 0.23% and the dialysis increased to 31.94 ± 0.31%. Protein concentration in crude enzyme was 1.12 ± 0.12 mg / mL and decreased significantly after dialysis to 0.06 ± 0.07 mg / mL. Purification by the addition of ammonium sulfate results in decreased enzyme protein content because the non-enzyme protein was precipitated.

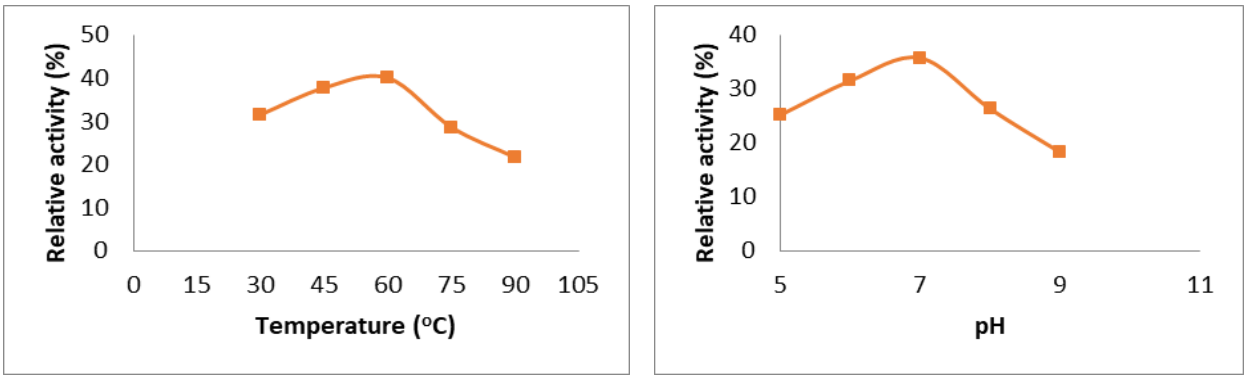
Table 2 also shows that the purity level of CCD enzyme from pomace increased after precipitation and dialysis compared to the relative activity value of the crude enzyme. These data indicate that purification treatments can enhance the relative activity of the CCD enzyme. In the study by Baldermann *et al*. (2009), the relative activity of the CCD enzyme from tea leaves was obtained at 60% using acetone for protein precipitation. Partial purification was also performed on quince fruit (*Cydonia oblonga*) through centrifugation, 85%

acetone precipitation, ultrafiltration (300 kDa, 50 kDa), and isoelectric focusing (pH 3-10) yielding a relative activity of 75%.

Table 2 Protein Content and Relative Activity of CCD

Sample	Protein concentration (mg/mL)	Relative Activity (%)
Crude enzyme	1.12±0.12	32.45
Precipitation	0.15±0.07	40.65
Dialysis	0.07±0.52	61.54

Determination of optimum temperature and pH was carried out on CCD enzymes on dialysis results. The determination of the optimum temperature was carried out by reacting the enzyme with the substrate at different temperatures (30, 45, 60, 75, 90 °C). From the perspective of protein structure, temperature affects the looseness and density of bonds in the enzyme's protein structure (Al-Baarri et al., 2018). Meanwhile, the determination of the optimum pH was performed by reacting the CCD enzyme with the β-carotene substrate under different pH conditions (5, 6, 7, 8, 9). The results for the optimum temperature and pH of the CCD enzyme can be seen in Figure 2.



(a) (b)

Figure 2. Temperature (a) and pH (b) dependence of carotenoid-cleavage activity of dialysis enzyme

The optimum temperature of the CCD enzyme was 60 °C (Figure 2). Until the optimum temperature is reached, the enzyme activity will rise in line with the temperature (Pertiwiningrum *et al*., 2017). At the optimum temperature, the conformation of the enzyme's protein structure is ideally positioned to bind the substrate and form the product, resulting in the highest activity.

Temperature changes also affect the bonds that maintain the enzyme's molecular conformation. Conformational changes impact the enzyme's active site, as certain heat conditions can break hydrogen bonds. Breaking one hydrogen bond can lead to the more manageable disruption of subsequent hydrogen bonds within the polypeptide chain, resulting in enzyme protein denaturation. The optimum temperatures of CCD in various plants were 45 °C in nectarines and starfruit and 70 °C in tea (Baldermann *et al*., 2005, 2010; Fleischmann, Studer, *et al*., 2002). These high optimum temperatures of carotenoid cleavage activity indicate that the enzyme can be an excellent alternative for the food industry, especially for several thermal processes.

The optimum pH value of b-carotene degradation activity was found to be 7 (Figure 2), while the optimum pH values for carotenoid-cleavage activity were reported in tea leaves as 7.4, in star fruit as 8.5, and quince in the range of 6– 9 (Baldermann *et al*., 2005; Fleischmann, Lutz-Röder, *et al*., 2002). At the optimum pH, the enzyme maintains the correct three-

dimensional conformation to bind the substrate effectively (Pratama et al., 2018). Under conditions outside the optimum pH, the enzyme's conformation begins to change, causing the substrate to be misaligned with the enzyme's active site. This misalignment hinders the catalytic process from proceeding optimally, resulting in reduced enzyme activity (Al-Baarri, Legowo, Wratsongko, et al., 2019).

3.3. Determination of protein molecular weight of CCD

Proteins that moved and halted at a specific migration distance during the electrophoresis process are indicated by the presence or absence of bands at that migration distance (Al-Baarri *et al*., 2024). After SDS-PAGE and commasie blue, seven protein bands were detected in the crude enzyme, six protein bands in precipitation with ammonium sulfate, and three bands in dialysis (Figure 3).

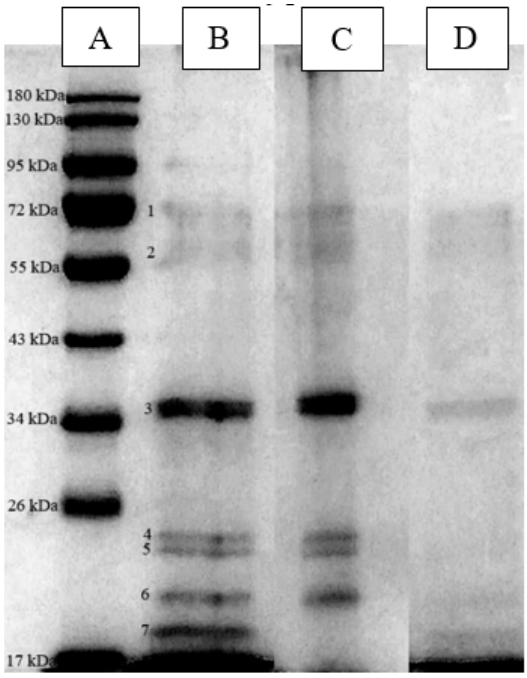


Figure 3. Polyacrylamide gel electrophoresis of the partially purified enzyme. (A) Marker (B) Crude enzyme (C) Precipited with ammonium sulfate (D) Dialysis enzyme

The molecular weight of the proteins was calculated from the calibration curve of the standard proteins (Rf value vs. mol.mass) used for the SDS PAGEs. Based on the results of colour intensity analysis using ImageJ, it can be seen that the dominant CCD enzyme band protein from the yellow passion fruit pomace is found at a molecular weight of 40 kDa. In previous studies, CCD enzymes isolated from *Arabidopsis thaliana* plants had a molecular weight of 66 kDa (Schwartz *et al*., 2004), and three bands were detected from quince fruit *(Cydonia oblonga)* which is 21.8 kDa, 23.9 kDa, and 25.9 kDa (Baldermann *et al*., 2005). The difference in molecular weight of CCD enzymes because the composition of CCD enzymes is different in each plant.

3.4 Identification of volatile compounds

From the identification results of yellow passion fruit pomace compounds using GC-MS, 18 types of volatile compounds were found with the highest number of components from terpene compounds (Table 3). Other groups of compounds are aldehydes, esters, alkene, phenol, naphthalene, and alcohol. Volatile compounds in the dominant yellow passion fruit pomace are ethyl hexanoate (15.67%) and 1-hexanol (14.07%).

Table 3 Volatile Compound in yellow passion fruit pomace

Group of Compound	Compound in yellow passion fruit pomace	% Relative Area
Aldehid	Heksanal	2.09
Terpene	α-gurjunene	6.09
	Veridiflorol	2.27
	Juniper champor	3.17
	Farnesol	6.38
	(+)-Aromadendrene	2.00
	Spathulanol	2.77
	Squalene	4.36
	Patchoulene	3.69
Alkena	Docosane	1.46
Alkohol	1-hexanol	14.07
Phenol	Phenol, 2 methoxy-3-(2 propenyl)	7.13
	3-allyl-6-methoxy phenol	1.41
Ester	ethyl hexanoate	15.67
	Butyl acetate	1.38
Naphtalene	1-naphtalenol	3.38
	1-naphtalenemethanol	1.99
Acid	Geranic acid	1.71

Ethyl hexanoate gives the aroma of sweet, floral, fruity. Hexanoate and acetate esters provide a fruity, sweet, citrus aroma. Esters, terpenes, alcohols, octanal compounds, dodecanol compounds and passion fruit scents, fruity, citric, and candy are scents detected in mature mature passion fruit (Janzantti *et al*., 2012; Janzantti & Monteiro, 2014; Leão *et al*., 2014). Terpenoid compounds are carotenoid derivatives that can give a distinctive flavour and aroma.

4. Conclusions

Carotenoid compounds identified in yellow passion fruit pomace: ζ-carotene, neoxanthin, β-carotene and luteinepoxide. The purified fraction showed carotenoid cleavage activity with β-carotene as substrate. The optimum temperature for enzymatic carotenoid-cleavage is 60 °C, whereas the optimum pH value of carotenoid cleavage activity is 7. Dominant volatile compounds in yellow passion fruit pomace are ethyl hexanoate and 1-hexanol.

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