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# Optimization of the use of cellulolytic enzyme preparation for the extraction of health promoting anthocyanins from black carrot using response surface methodology

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ABSTRACT

Anthocyanin-rich extracts from black carrots are being considered as a candidate replacer for the red colour in processed foods. The present investigation optimizes the extraction of anthocyanins with high phenolic content and low degradation parameters from black carrots using a cellulolytic multi-enzyme preparation known as Viscozyme. The optimized conditions for Viscozyme using a Box-Behnken design (BBD) of response surface methodology (RSM) were as follow: temperature (50.2 °C), extraction time (58.4 min) and enzyme concentration (0.20%). The predicted value of anthocyanins content was 1380 mg/L, which was near to the optimized experimental value of 1375 mg/L. The extracted anthocyanins based on above mentioned conditions exhibited the lowest degradation parameters such as degradation index (DI) of (0.86), browning index of (BI) (1.31) and were characterized with cyanidin 3-sinapoylxylosylglucosylgalactoside as being the most abundant. The findings clearly reveal that Viscozyme-assisted extraction (VAE) is the best approach for extracting superior quality extracts from black carrots with high anthocyanin and other phenolic component concentrations.

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**Abbreviations**

| | |
|---|---|
| TAC | total monomeric anthocyanins content |
| TFC | total flavonoids content |
| TPC | total phenolics content |
| AOC | antioxidant capacity |
| CD | colour density |
| PC | polymeric colour |
| BI | browning index |
| DI | degradation index |
| FRAP | ferric reducing antioxidant power |
| GAE | gallic acid equivalents |
| ANOVA | analysis of variance |
| GRAS | generally recognized as safe |
| RSM | response surface methodology |
| VAE | viscozyme-assisted extraction |
| PPO | polyphenol oxidase |
| CE | conventional extraction |
| BBD | Box-Behnken design |
| TFA | trifluoroacetic acid |
| HPLC | high-performance liquid chromatography |
| QE | quercetin equivalents |
| EC | enzyme concentration |
| CCG | cyanidin 3-coumarylxylosylglucosylgalactoside, |
| CXG | cyanidin 3-xylosylglucosylgalactoside; |
| CSG | cyanidin 3-sinapoylxylosylglucosylgalactoside; |
| CFG | cyanidin 3-feruloylxylosylglucosylgalactoside; |
| CXGal | cyanidin 3-xylosylgalactoside; |
| CTs | condensed tannins |

## 1. Introduction

Black carrot (*Daucus carota* ssp. sativus var. atrorubens Alef.) is an industrial crop, and a known source of commercial anthocyanin (E163) concentrate used as a pigment in the food industry. Anthocyanins are categorized as natural food colorant with red, pink and purple hues and have a 'Generally Recognized as Safe' (GRAS) status. Presently, Turkey is a global leader in black carrot production with an increasing tonnage every year due to the rapidly increasing demand around the world. Anthocyanins have been widely used in industries, including food, cosmetics, and pharmaceuticals due to their natural colorant, antioxidant and therapeutic properties (Kumar et al., 2019a). The need for natural colourants is increasing globally due to the rising requirement for naturally derived ingredients and colouring agents with a 'clean label' (Kumar et al., 2019a; Kumar et al., 2019b). Black carrot concentrate has just been approved by the US FDA (Food and Drug Administration) as a substitute for carmoisine in foods. As a result, anthocyanins extracted from black carrots have a high commercial potential, and optimizing the extraction procedure is critical. Enzyme-assisted processing for extracting bioactive chemicals from plants has received a lot of attention in recent years because of its improved efficiency, ease of use, and environmental friendliness. Furthermore, the enzymes are non-toxic and a wonderful example of green technology (Chavez-Gonzalez et al., 2016; Kaur et al., 2016; Manzoor et al., 2021). Extraction of anthocyanins from black carrots is challenging process as straight pressing does not completely extract anthocyanins, because the pigments are tightly bound to the cell wall matrix (Khandare et al., 2011; Weber et al., 2017; Kumar et al., 2019a; Dini et al., 2020). Enzymes offer the best solutions for maximum recovery of anthocyanins (Casas & Dominguez, 2017; Gligor et al., 2019; Türker & Doğan, 2021) and have several merits over conventional extraction methods. The reaction conditions are mild with lower extraction temperature and require a shorter time period, less number of steps, and a high substrate specificity resulting in targeted extraction to yield high-quality pure extracts (Banerjee et al., 2017; Kamiloglu et al., 2017). Enzyme preparations like cellucast, Viscozyme and pectinase in their pure or crude form can be used to disintegrate plant matrices and assist in the extraction of phenolics, including anthocyanins (Cinar, 2005; Kumar et al., 2019b).

Viscozyme is a cellulolytic multi-enzyme formulation containing various carbohydrases like xylanase, hemicellulase, β-glucanase, cellulase, and arabanase. Viscozyme can be effectively employed to improve the yield and quality of black carrot juice (Kaur et al., 2016). Hemicellulases, cellulases, pectin methyl esterases and protopectinases present in the commercially available enzyme cocktails act in synergy and improve the extraction efficiency (Muniglia et al., 2014; Danalache et al., 2018; Parada & Dominguez, 2018). Therefore, Viscozyme was used in the current study to maximize the extraction of anthocyanins from black carrots. The researchers concluded that the enzyme-assisted extraction is the perfect method to obtain biologically active anthocyanins from various plant matrices (Lotfi et al., 2015; Kamiloglu et al., 2017; Kanha et al., 2019; Swer & Chauhan, 2019; Zhang et al., 2020). Optimization of the enzyme concentration and incubation time for extraction process is very critical so as to obtain a high content of anthocyanins and other phenolics in the extracts. The obsolete approach of single factor at a time for optimization of enzyme-based extraction is time-consuming and disregards the relationships between various input factors. Response surface methodology (RSM) is, thus, a modern strategy to optimize the process parameters for getting a stochastic response (Kamiloglu et al., 2017; Pompeu, 2009) and is currently used to optimize various factors in VAE and maximize the yield of bioactive compound extractions from plant material. A Box-Behnken design (BBD) is a multivariable optimization technique relying on three-level incomplete factorial designs and is used for a second-order response surface model (Kaur et al., 2016). Compared to other experimental designs, this design was found to be superior to the quadratic model because of its higher efficiency and lower cost of experimentation. Anthocyanin rich extracts from black carrots is being considered as a candidate replacer for red colour in processed foods. However, the stability of anthocyanins is the main issue and during extraction, monomeric anthocyanins may degrade to form polymeric brown pigments, thereby raising the browning and degradation index. Hence, browning parameters need to be minimized to obtain high quality extracts. The main challenge in the food industry is to search for improved processing technologies and stabilization methods to better control the loss of anthocyanins and to safeguard such promising compounds from degradation in order to potentially increase their bioavailability (Gullon et al., 2017; Belwal et al., 2018).

Consequently, the present investigation was conducted to study the impact of Viscozyme in obtaining quality anthocyanin- and phenolic-rich extracts with a low browning index and polymeric colour, as well as to compare viz. VAE and conventional extraction (CE) for the extraction of anthocyanins, total phenolics, and flavonoid compounds from black carrots. This is the first study conducted on maximizing of anthocyanin extraction with high antioxidant activity and low degradation parameters using Viscozyme. The study was conducted with an intent to develop a protocol of VAE for high anthocyanins yield and lower degradation parameters, and developing an optimum process to guarantee high yield of anthocyanins extraction from black carrot. This process will help the food industry to obtain a high-quality extract with increased stability. This research is a continuation of previous work (Kaur et al., 2016) with due emphasis on the degradation and stability parameters related to anthocyanins.

## 2. Material and methods

### 2.1. Raw material

Freshly harvested black carrots (Pusa Asita cultivar) of medium size were procured in the year 2015, from the fields in the Biochemistry division, Indian Agricultural Research Institute, New Delhi. Carrots were thoroughly cleaned with running tap water to eliminate attached dirt particles.

### 2.2. Extraction experiments

The washed carrots were then peeled with a peeling knife (stainless-steel) and chopped into small slices followed by crushing in a domestic blender. The prepared samples were heated at 90 °C for 1 min followed by rapid cooling to 40 °C to inactivate the inherent polyphenol oxidase (PPO). A sample portion (10 g) was transferred to a flask for the isolation of bioactive anthocyanins and other phenolics at different enzyme concentrations, temperature and extraction time. A three-level and three-variable BBD with Viscozyme concentration (A: 0.1–0.3%), time (B: 30–90 min) and temperature (C: 40–60 °C) for enzyme-assisted extraction. Viscozyme was supplied by Sigma-Aldrich (Oakville, Canada). The process variable and ranges were selected on the basis of the preliminary experiments, which were carried out to explore the effect of individual input factor. The extracts were filtered through Whatman #1 filter paper and concentrated at 40 °C using a rotary evaporator (Heidolph, Schwabach, Germany) to increase their storage stability. Amberlite XAD 16HP (Sigma-Aldrich) was used to remove sugars from the extracts. The crude sample extract was applied onto the column washed with deionized water. The water was used for removing sugars from extract and then phenolic compounds were isolated from the column using methanol. The resulting phenolic compound solution was stored in the dark at 4 °C for subsequent analysis. This solution was analyzed for anthocyanins, total phenolics, total flavonoids, CD, AOC, total phenols and degradation parameters (DI, BI and PC). For CE, 10 g of the black carrot mash was used for extraction of anthocyanins and other phenolic components with citric acid acidified water as the solvent. Prior to shaking, the pH was adjusted to 3.0, verified, and if necessary, re-adjusted after 15–30 min. Similarly, water acidified with acetic acid, citric acid, acetic acid + sulphur dioxide at pH 3 was also used for extraction of anthocyanins from black carrots. The extracts obtained from the CE method were analyzed for anthocyanin yield and compared with those from VAE (Kaur et al., 2016; Kumar et al., 2019b; Kumar et al., 2020).

### 2.3. Analytical methods

The analytical methods were performed according to our previous study on black soybean seed coats (Kumar et al., 2019a). By using spectrophotometric methods, TAC (total anthocyanin content), TFC (total flavonoids content), TPC (total phenolics content) were determined. Colour density has been taken as one of the parameters for the optimization, which is a very important parameter depicting the stability of anthocyanins. Anthocyanin degradation in the polyphenolic extract is monitored using PC (polymeric colour), which provides insight into the presence of polymeric components in the extract. By measuring the absorbance of bisulfate-treated samples at 420 nm, BI (browning index) was determined. Using the ratio of 420 nm–520 nm absorbance, DI (degradation index) was calculated. AOC (antioxidant capacity) was determined by FRAP (ferric reducing antioxidant power) assay.

### 2.4. Anthocyanins identification from black carrots extract by HPLC

The anthocyanins rich extract from black carrots, obtained by VAE, was analyzed using a Waters HPLC system comprising an auto injector, quaternary pump and photodiode array as detector (Waters Corp., Milford, MA., U.S.A) with an ODS Hypersil column (250 × 4.6 mm, 5-μm) (Thermo Electron Corporation). Sample aliquots (2 mL) were filtered through a nylon filter (0.45-μm) before injection into the HPLC system. Water with 0.1% TFA (solvent A) and a ratio of water (53): acetonitrile (46): TFA (1) (solvent B) were used as the mobile phases. A wavelength of 520 nm was selected for the identification of the anthocyanins. The analysis of the peak areas was performed with "Empower 2" software.

### 2.5. Box-Behnken (BBD) design and statistical analyse

Statistical analyse and BBD for VAE were performed according to Kumar et al (2018b). The experimental design and statistical analysis were performed using Design Expert 12.0 software (Stat-Ease Inc., USA, licensed to ICAR-CMFRI). The analysis and design were carried out using Stat-Ease software. The input variables and their ranges were selected on the basis of preliminary results obtained from single factor experiments. The input factors (enzyme concentration, time, temperature) and responses (TAC, TFC, TPC, AOC, colour parameters and degradation parameters) were subjected to ANOVA and regression analysis to assess the significance of the performed model. The significance of linear, interactive and quadratic terms was also established using regression and ANOVA analysis.

## 3. Results

Enzyme-based extraction is considered as a green, rapid, efficient and economical method for extraction of anthocyanins and other phenolics from plant matrices (Kumar et al., 2020). The extraction parameters were optimized by Viscozyme and CE methods employing acidified water with citric acid and acetic acid. Acidified water composed of acetic acid + sulphur dioxide was carried out as a reference method for comparing the efficiency of VAE. Cellulase (Kumar & Dahuja, 2019) and pectinase (Kumar et al., 2017) have already been used for anthocyanins extraction from black carrots and have given promising results. The black carrot mash was subjected to Viscozyme and CE for the development of anthocyanins and phenolic-rich extracts.

### 3.1. Viscozyme-assisted extraction (VAE) of bioactives from black carrots

Experimental results acquired from conducting 17 trials with 5 replications of the centre points to measure the pure error were executed using a BBD as shown in Table 1. The highest anthocyanin (1488 mg/L) yield was acquired using Viscozyme in comparison to the previous study where cellulase (Kumar & Dahuja, 2019) and pectinase (Kumar et al., 2017) were employed. The extracts obtained by VAE were then analyzed for TAC, TFC, TPC, DI, BI, PC, CD, and AOC by FRAP. Dependent variable values were found between the following: TAC (190–1488 mg/L), TFC (420–3122 mg QE/L), TPC (84–502 mg GAE/100 mL), DI (0.73–14.1 units), BI (1.1–18.23 units), PC (1.15–30.33 units), CD (4.97–75.25 units) and FRAP (2.06–25.61 μmol TE/mL). Different experimental conditions govern various responses, which in turn influence the contents of various bioactive components (TAC, TFC, TPC), like degradation attributes (DI and BI), colour attributes (PC and CD) and in vitro AOC (FRAP). Thus, in order to select the most pertinent model for the different 8 responses; i.e., TAC, TFC, TPC, DI, BI, PC, CD, and FRAP, the data of Table 1 was incorporated in a second-order model with different terms (quadratic, interactive and linear) by ANOVA and regression analysis to verify the appropriateness of the model across other tested models. The fitted model's summary reports (Table 2) displays that the second order model had a higher $R^2$ (>0.90), signifying that the regression models could appropriately describe fitness of the model. Additionally, the adjusted- $R^2$ values (second order model) were greater than 0.828 for all the responses, validating the significance of the fitted model. The model adequacy confirmed using the data from the ANOVA (Table 2) established that second order model was most

**Table 1**
Three-level and three-variable Box-Behnken design with Viscozyme concentration (A: 0.1–0.3%), time (B: 30–90 min) and temperature (C: 40–60 °C) and experimental data of TAC, TPC, TFC FRAP, CD, PC, BI and DI for Viscozyme-assisted extraction.

| Run | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 | Response 3 | Response 4 | Response 5 | Response 6 | Response 7 | Response 8 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A: EC (%) | B: Time (minutes) | C: Temp (°C) | TAC (mg/l) | TPC (mg GAE/100 mL) | TFC (mg QE/l) | FRAP (mol TE/ml) | CD (units) | PC (units) | BI (units) | DI (units) |
| 1 | 0.3 | 60 | 40 | 715.83 | 211.13 | 1822.22 | 11.79 | 27.92 | 5.55 | 2.12 | 1.53 |
| 2 | 0.2 | 90 | 60 | 278.31 | 130.47 | 420.00 | 4.23 | 7.33 | 30.33 | 18.23 | 14.10 |
| 3 | 0.1 | 60 | 40 | 1022.53 | 293.93 | 1848.33 | 16.56 | 43.52 | 7.65 | 4.3 | 3.23 |
| 4 | 0.2 | 90 | 40 | 752.37 | 247.67 | 1607.22 | 13.20 | 35.79 | 25.53 | 17.34 | 8.40 |
| 5 | 0.2 | 30 | 40 | 189.81 | 84.27 | 610.00 | 2.06 | 4.97 | 16.11 | 9.68 | 7.43 |
| 6 | 0.2 | 60 | 50 | 1337.58 | 449.93 | 3033.33 | 21.99 | 65.26 | 2.67 | 1.5 | 1.05 |
| 7 | 0.1 | 30 | 50 | 627.88 | 186.93 | 1986.67 | 9.58 | 22.68 | 10.09 | 5.67 | 2.30 |
| 8 | 0.2 | 60 | 50 | 1311.98 | 454.93 | 3042.22 | 22.71 | 65.47 | 2.74 | 1.1 | 0.73 |
| 9 | 0.1 | 90 | 50 | 723.06 | 225.53 | 1653.89 | 12.03 | 28.64 | 22.94 | 12.45 | 9.59 |
| 10 | 0.3 | 90 | 50 | 783.18 | 255.07 | 2309.44 | 13.07 | 32.99 | 14.21 | 10.23 | 7.86 |
| 11 | 0.1 | 60 | 60 | 812.12 | 259.67 | 1511.67 | 13.12 | 30.91 | 4.75 | 2.67 | 1.36 |
| 12 | 0.3 | 30 | 50 | 332.31 | 96.73 | 1482.22 | 5.44 | 10.49 | 4.92 | 2.2 | 1.59 |
| 13 | 0.2 | 60 | 50 | 1324.22 | 457.07 | 2963.33 | 22.63 | 65.37 | 4.32 | 1.22 | 0.83 |
| 14 | 0.2 | 60 | 50 | 1488.43 | 467.07 | 3077.22 | 21.95 | 65.17 | 2.54 | 2.56 | 1.87 |
| 15 | 0.2 | 30 | 60 | 634.56 | 176.67 | 1318.89 | 9.22 | 26.24 | 8.12 | 4.56 | 2.43 |
| 16 | 0.2 | 60 | 50 | 1476.35 | 502.37 | 3122.45 | 25.61 | 75.25 | 1.15 | 1.78 | 1.06 |
| 17 | 0.3 | 60 | 60 | 836.06 | 262.67 | 1820.56 | 14.02 | 31.61 | 2.61 | 1.13 | 0.76 |

Where, A = enzyme concentration, B = Time, C = temperature, TAC = total anthocyanin content, TFC = total flavonoid content, TPC = total phenolic content, AOC = antioxidant capacity, CD = colour density, PC = polymeric colour, BI = browning index, DI = degradation index, EC = enzyme concentration (Kumar et al., 2016, Ph.D. thesis).

**Table 2**
Model summary statistics- ANOVA (for significant values), Regression coefficient, coefficient of determination ($R^2$) and value of F test statistics of the second order polynomial models for the bioactive compounds, antioxidant capacity, colour attributes and degradation attributes.

| | Regression coefficients | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Degree of freedom | TAC | TPC | TFC | FRAP | CD | PC | BI | DI |
| Intercept | 9 | 1387.71 (36.04) | 466.27 (9.02) | 3047.71 (32.97) | 22.98 (0.57) | 67.3 (1.85) | 2.68 (0.91) | 1.63 (0.46) | 1.11 (0.26) |
| Linear | | | | | | | | | |
| A | 1 | −64.78* (28.49) | −17.56** (7.13) | 54.24* (26.06) | −0.87* (0.45) | −2.84* (1.46) | −2.27* (0.72) | −1.18** (0.37) | −0.59** (0.2) |
| B | 1 | 94.05** (28.49) | 39.27*** (7.13) | 74.1** (26.06) | 2.03*** (0.45) | 5.05** (1.46) | 6.72*** (0.72) | 4.52*** (0.37) | 3.27*** (0.2) |
| C | 1 | −14.94 (28.49) | −0.94 (7.13) | −102.08*** (26.06) | −0.38 (0.45) | −2.01 (1.46) | −1.13 (0.72) | −0.86* (0.37) | −0.24 (0.2) |
| Interactive | | | | | | | | | |
| AB | 1 | 88.92* (40.29) | 29.93** (10.08) | 290*** (36.86) | 1.29* (0.63) | 4.14* (2.07) | −0.89 (1.02) | 0.31 (0.52) | −0.26 (0.29) |
| AC | 1 | 82.66* (40.29) | 21.45* (10.08) | 83.75* (36.86) | 1.42* (0.63) | 4.07* (2.07) | −9.70E-03 (1.02) | 0.16 (0.52) | 0.27 (0.29) |
| BC | 1 | −229.7*** (40.29) | −52.4*** (10.08) | −474.03*** (36.86) | −4.03*** (0.63) | −12.44*** (2.07) | 3.2** (1.02) | 1.5** (0.52) | 2.67*** (0.29) |
| Quadratic | | | | | | | | | |
| $A^2$ | 1 | −194.12*** (39.27) | −89.06*** (9.83) | −213.99*** (35.93) | −3.13*** (0.62) | −14.35*** (2.02) | −2.26* (0.99) | −1.95*** (0.5) | −1.07*** (0.28) |
| $B^2$ | 1 | −576.99*** (39.27) | −186.14*** (9.83) | −975.66*** (35.93) | −9.82*** (0.62) | −29.25*** (2.02) | 12.62*** (0.99) | 7.95*** (0.5) | 5.3*** (0.28) |
| $C^2$ | 1 | −346.96*** (39.27) | −120.36*** (9.83) | −1083.02*** (35.93) | −5.98*** (0.62) | −19.47*** (2.02) | 4.72*** (0.99) | 2.87*** (0.5) | 1.68*** (0.28) |
| Residual | 7 | | | | | | | | |
| Lack of fit | 3 | 0.6145 (ns) | 0.5578 (ns) | 0.2149 (ns) | 0.8271 (ns) | 0.6042 (ns) | 0.0538 (ns) | 0.0576 (ns) | 0.2005 (ns) |
| Pure error | 4 | | | | | | | | |
| Total | 16 | | | | | | | | |
| R-Squared | | 0.983 | 0.9905 | 0.9966 | 0.9857 | 0.9848 | 0.9776 | 0.9855 | 0.991 |
| Adj R-Squared | | 0.9612 | 0.9783 | 0.9923 | 0.9673 | 0.9652 | 0.9487 | 0.9669 | 0.9794 |
| Pred R-Squared | | 0.8918 | 0.9339 | 0.9639 | 0.94 | 0.9012 | 0.6975 | 0.8063 | 0.9013 |
| Adeq Precision | | 19.947 | 25.689 | 45.289 | 22.133 | 20.221 | 17.388 | 21.364 | 28.794 |
| CV% | | 9.35 | 7.2 | 3.73 | 9 | 11 | 20.76 | 17.79 | 14.78 |

Where, A = enzyme concentration, B = Time, C = temperature, AB = enzyme concentration × time, AC = enzyme concentration × temperature, BC = Time × Temperature, TAC = total anthocyanin content, TPC = total phenolic content, TFC = total flavonoid content, FRAP = ferric reducing antioxidant power, CD = colour density, PC = polymeric colour, BI = browning index, DI = degradation index, and $A^2$, $B^2$ and $C^2$ are representing the quadratic terms for enzyme concentration, time, temperature respectively and values shown are regression coefficient of respective term. Figures in parenthesis denotes standard error. *Significant at $p < 0.1$, **significant at $p < 0.05$, ***significant at $p < 0.01$ rest other values are non-significant. TAC = total anthocyanin content, CV = Co-variance, Adj R-squared = Adjusted R-squared, Pred R-squared = Predicted R-squared, Adeq Precision = Adequate Precision (Kumar et al., 2016, Ph.D. thesis).

significant and could appropriately signify real relationships among the significant parameters and responses with lower p-value ≤ 0.05 between the experimental and the predicted values. Thus, the results indicated that the second order model concurred with findings of the experiment, suggesting the appropriateness of the final model. Consequently, second order modelling was selected for analysing the results. Therefore, the responses generated from a BBD were examined through regression models. On the basis of ANOVA (Table 2) fitted second order models for the responses of TAC, TFC, TPC, DI, BI, PC, CD and FRAP, as a function of enzyme concentration (EC) (A), time (B), temperature (C) was obtained. In terms of coded variables, the quadratic regression model demonstrates the action of various operational conditions on different responses, and is presented as equations in Table 3. Table 3 represents the optimized values for the high yields of total phenolics, total flavonoids, and anthocyanins together with highest AOC, colour properties and less DI, BI and PC.

### 3.1.1. Total monomeric anthocyanins content (TAC)

The effect of 3 input factors on TAC was significant ($p < 0.01$) and is demonstrated through the quadratic regression equation (Table 3). The concentration of enzyme, temperature, and time (Table 2) exhibited significant linear effects in time (B) and EC (A), while the interactive effects are significant in BC, AC, AB and quadratic effects in $C^2$ and $B^2$ for TAC. The relationship between the three extraction parameters and TAC was explained by the response surface plots (Figs. 1.1–1.3). The effect of mutual interaction between EC and incubation time (B) is shown in Fig. 1.1. At a minimum incubation time (30 min), a decline in TAC (390.2 mg/L) was noticed with a raise in EC (up to 0.3%). The maximum value of TAC (1389 mg/L) was found at an EC of 0.2% and time of 60 min. The effect of mutual interaction between EC (A) and temperature (C) on TAC is depicted in Fig. 1.2. It indicates that the maximum TAC (1386 mg/L) was obtained at an EC of 0.2% and 50 °C. Fig. 1.3 presents the effect of mutual interaction between time and temperature. A minimum TAC (179 mg/L) was obtained at short time (30 min) and temperature (40 °C). The interaction between B and C was highly significant with $p < 0.01$. At 50–60 min and 45–55 °C, the highest value of TAC (1388 g/L) is determined in response curve (Fig. 1.3). It is evident from the three-dimensional response curve that the values of temperature (45–55 °C), time (50–60 min) and EC (0.18–0.22%) could achieve the highest value of TAC (see Fig. 2).

**Table 3**

The equations of regression models for responses in black carrot extracts in case enzyme-assisted extraction by Viscozyme and comparison of Viscozyme-assisted extraction and conventional extraction (CE) of anthocyanins (Kumar et al., 2016, Ph.D. thesis).

| Sr. No. | Response | Models | Optimized condition | Yield of TAC (mg/l) |
|---|---|---|---|---|
| Enzyme assisted Extraction by Viscozyme | | | | |
| 1. | TAC | $1387.71–64.77A + 94.04B − 14.93C + 88.92AB + 82.65AC − 229.70BC − 194.11A^2 − 576.98B^2 − 346.96C^2$ | EC = 0.201%<br>Time = 58.40 min<br>Temp = 50.18 °C | TAC = 1375.43 ± 38.99 |
| 2. | TPC | $466.27–17.5A + 39.26B − 0.94C + 29.93AB + 21.45AC − 52.40BC - 89.06A^2 − 186.14B^2 − 120.36C^2$ | | |
| 3. | TFC | $3047.71 + 54.23A + 74.09B − 102.08C + 290AB + 83.75AC − 474.02BC − 213.99A^2 − 975.66B^2 − 1083.02C^2$ | | |
| 4. | FRAP | $22.97–0.87A + 2.02B − 0.37C + 1.29AB + 1.42AC − 4.03BC − 3.12A^2 − 9.82B^2 − 5.97C^2$ | | |
| 5. | CD | $67.3014–2.84A + 5.04B − 2.01C + 4.13AB + 4.07AC − 12.43BC − 14.346A^2 − 29.25B^2 −19.46C^2$ | | |
| 6. | PC | $2.68–2.26A + 6.72B − 1.12C − 0.88AB − 0.0097AC + 3.19BC − 2.26A^2 + 12.61B^2 + 4.72C^2$ | | |
| 7. | BI | $1.63–1.17A + 4.51B − 0.85C + 0.31AB + 0.16AC + 1.50BC −1.94A^2 + 7.95B^2 + 2.86C^2$ | | |
| 8. | DI | $1.10–0.59A + 3.27B − 0.24C − 0.25AB + 0.27AC + 2.67BC − 1.07A^2 + 5.2B^2 + 1.68C^2$ | | |
| CE | | | | |
| 1. | Water acidified with citric acid | | pH = 3 | TAC = 323.40 ± 21.73 |
| 2. | Water acidified with acetic acid | | pH = 3 | TAC = 255.49 ± 10.43 |
| 3. | Water acidified with acetic acid + Sulphur di oxide | | pH = 3 | TAC = 444.75 ± 9.2 |

### 3.1.2. Total phenolics content (TPC)

The linear effect of A and B was found to be significant, while C was non-significant (Table 2). The mutual effects of interaction between BC ($p < 0.0001$) and AB ($p < 0.05$) were significant along with all the quadratic terms ($p < 0.0001$). The relationship between the three extraction parameters and TPC was explained by the response surface plots (Supplementary information: Fig. S1.1-S1.3). Fig. S1.1 displays the interaction effect between time and EC ($p < 0.05$). At an incubation time of 50–70 min and EC (0.19–0.2%), the highest TPC value of 466.7 mg GAE/100 mL was determined. Fig. S1.2 demonstrates the interaction between EC and temperature on TPC. The figure shows that near the centre of the response curve at EC (0.2%) and 50 °C, a maximum value of TPC (466,0 mg GAE/100 mL) was observed. Contour plots with elliptical or circular shapes illustrate the importance of mutual interaction between the variables (Muralidhar et al., 2001). Fig. S1.3 displays the outcome of the interaction between incubation time and temperature on TPC. The highest phenolic yield (88.0 mg GAE/100 mL) was observed at a minimum time (30 min), and temperature (40 °C). This interaction between C and B was found to be significant at the level of $p < 0.01$. At 45–55 °C and 50–60 min the maximum value of TPC (466.0 mg GAE/100 mL) was shown by the response curve. It is evident from the three-dimensional response curve that the values of temperature (45–55 °C), time (50–70 min) and EC (0.19–0.20%) could achieve the highest value of TPC.

### 3.1.3. Total flavonoids content (TFC)

The deviation in the content of flavonoids as the function of various input parameters was explained by the regression equation of TFC at the significance level of $p < 0.0001$. For the extraction of flavonoid, it was noted that the linear factors of time (B) and temperature (C) were significant. However, all interactive and quadratic effects were found significant, where, AB, BC, $A^2$, $B^2$ and $C^2$ were found highly significant ($p < 0.001$). In accordance with quadratic and linear coefficients, the positive coefficient for A and B, and negative coefficient for C, $A^2$, $B^2$ and $C^2$ reveal that TFC increased with time (30–90 min) and EC (0.1–0.3%), but decreases with an increase in temperature, time and Viscozyme concentration irrespective of range of input factor. A similar study indicated that flavonoids yield decreases by prolonging the extraction time due to the disintegration of bioactive compounds (Wang et al., 2010). Figs. S2.1–S2.3 depict the relationships between the three extraction parameters and TFC by response surface plots. A similar trend was observed for TFC on surface curves to those of TAC and TPC.

### 3.1.4. Antioxidant capacity (AOC)

The variation in AOC as the function of various extraction parameters is explained by the regression equation of AOC at the significance level of $p < 0.0001$. With respect to AOC, temperature, time, and EC were significant for B ($p < 0.01$), and non-significant for C with $p > 0.1$. All quadratic ($A^2$, $B^2$, $C^2$) and interactive effects (BC) were found to be

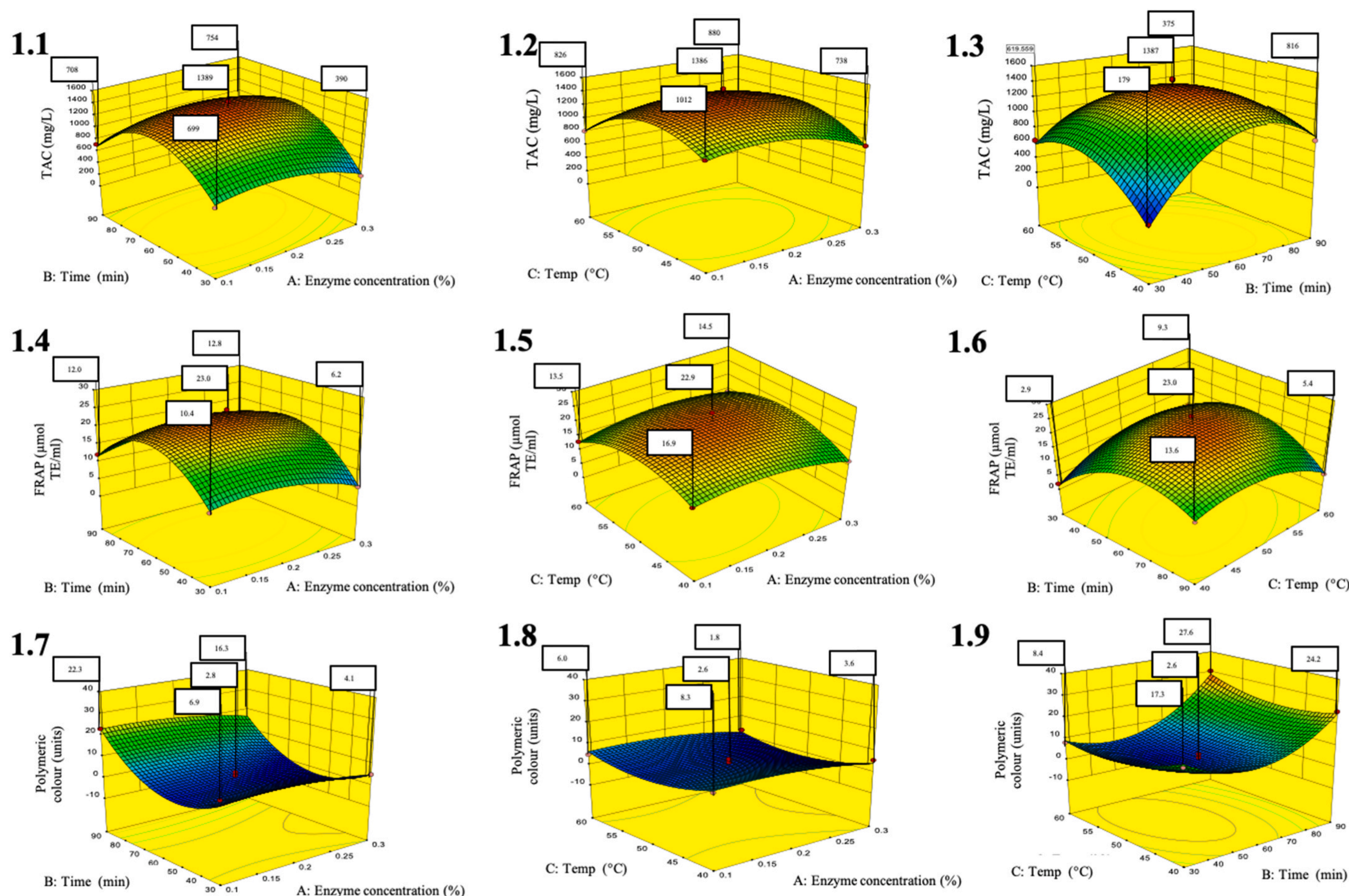


**Fig. 1.** Response surface analysis of black carrots for the effect of EC and time, EC and temperature, time and temperature on TAC, antioxidant potential using FRAP and polymeric colour using Viscozyme. Where, TAC = total anthocyanins content, FRAP = ferric reducing antioxidant power, Trolox Equivalents, PC = polymeric colour, EC = enzyme concentration.

significant at $p < 0.001$. The relation between three extraction parameters and AOC using FRAP was demonstrated by response surface plots (Fig. 1.4–1.6). The mutual interaction response curves between time, Viscozyme concentration, and temperature show a similar pattern as those for TAC, TPC and TFC as AOC of the extract, which is due to the bioactive content of the extract. The three-dimensional response curve indicates that the values of temperature (45–55 °C), time (50–60 min), and EC (0.15–0.25 percent) can give a maximum AOC value.

### 3.1.5. *Colour density (CD)*

The variation in CD as a function of the various extraction parameters was explained by the regression equation of CD at the significance level of $p < 0.0001$. For CD, temperature, time, and EC (Table 2) were significant for B ($p < 0.05$) and non-significant for C with $p > 0.1$. All the quadratic ($A^2$, $B^2$, $C^2$) and interactive effects (BC) were significant at $p < 0.001$. Fig. S3.1–S3.3 depict the relationship between the three extraction parameters and CD by response surface plots. It is evident from the three-dimensional response curve that the values of temperature (50–55 °C), time (50–60 min) and EC (0.2%) could achieve the highest value of CD.

### 3.1.6. *Polymeric colour (PC)*

The variation in PC as the function of the various extraction parameters was explained by the regression equation of PC at the significance level of $p < 0.0001$. For PC, the temperature, time, and EC (Table 2) were highly significant for B ($p < 0.0001$), A ($p < 0.05$) and non-significant for C. Interaction between B and C was found statistically significant ($p < 0.05$) as well as the other quadratic effects $C^2$ ($p < 0.01$) and $B^2$ ($p < 0.0001$) were statistically significant. Fig. 1.7–1.9 show the relationship between the three extraction parameters and PC by response surface plots. Fig. 1.7 demonstrates the effect of interaction between EC and time on PC. A lower value of PC of the juice is considered desirable, as the juice with a higher PC is related to greater degradation of anthocyanins. The value of PC was at least 2.8 units for a wide range of incubation time (50–60 min), and EC (0.1–0.3%). Incubation of 60 min, and an EC of 0.2% gave the highest value of PC (22.30 units). B and A showed a statistically non-significant ($p > 0.1$) interaction. Fig. 1.8 indicates the effect of mutual interaction of EC (A) and temperature (C) on PC. At an EC of 0.1% and 40 °C, the PC displays the highest value of 8.29 units. The lowest value of PC (1.85 units) was found at an EC of 0.3% and temperature of 60 °C. Fig. 1.9 shows the effect of mutual interaction of time (B) and temperature (C) on PC. The highest value of PC (2.58 units) was observed at the time (40–60 min) and temperature (50–60 °C). A significant interaction was observed between C and B ($p < 0.01$). The highest value of PC was found at an incubation time of 90 min and 60 °C through the response curve. It is evident from the three-dimensional response curve that the values of temperature (50–60 °C), time (50–60 min) and EC (0.1–0.3%) could achieve the highest value of PC.

### 3.1.7. *Browning index (BI)*

The variation in BI as a function of the various extraction parameters was explained by the regression equation of BI at the significance level of $p < 0.0001$ (Table 3). The temperature, time and EC were statistically significant for linear effects B ($p < 0.0001$) and A ($p < 0.05$) for BI. The incubation time exhibited the highest level of statistical significance. A

study indicated that extraction time affects colour degradation and increases the BI (Tchabo et al., 2015; Tiwari et al., 2010). Only BC was found to be significant at $p < 0.05$ whereas the interaction between AC and AB was non-significant ($p > 0.1$) whereas the quadratic terms were highly significant ($p < 0.001$). A study demonstrated that cavitation determines numerous biological, chemical and physical reactions by acting as a catalyst, augmenting the diffusion rate, dispersing the aggregates or disintegrating susceptible particles, which could increase the value of BI (Sala et al., 1995). Fig. S4.1–S4.3 depict the relationship between three extraction parameters and BI by response surface plots.

#### 3.1.8. Degradation index (DI)

The variation in DI as a function of the various extraction parameters was explained by the regression equation of DI at a significance level of $p < 0.0001$ (Table 3). Two linear terms B ($p < 0.0001$) and A ($p < 0.05$) were found significant whereas C was non-significant ($p > 0.1$). The interaction was non-significant ($p > 0.1$) between AC and AB while found significant ($p < 0.0001$) between B and C. In addition, all of the quadratic terms were found significant ($p < 0.01$). Fig. S5.1–S5.3 shows the relationship between three extraction parameters and DI by response surface plots.

### 3.2. Optimization of extraction parameters and validation of the model

RSM is a statistical tool for experimental modelling, reducing the experimental runs, optimization of processes and also for checking the interaction between the processes. This helps to identify the most favourable response for the specific extraction strategy (Nishad et al., 2021). RSM was aimed to identify extraction conditions with the highest TAC, TFC, TPC, CD, AOC and the lowest DI, BI, PC. The ideal values of chosen variables were acquired by solving the equation through a trial version of Design-Expert 9 software. Optimal parameters for extraction were temperature (50.2 °C), extraction time (58.4 min) and EC (0.20%). The greatest predicted values of TAC, TFC, TPC, CD and AOC were 1380 mg/L, 3039 mg QE/L, 463 mg/100 mL, 66.89 units, and 22.9 μmol TE/mL respectively, while the lowest predicted values of DI, BI, PC were 0.93 units, 1.38 and 2.31 respectively. Under optimum conditions, TAC was 1375, TFC was 3053 mg QE/L, TPC was 460 mg GAE/100 mL, CD was 67 units, FRAP was 23.3 μmol TE/mL, DI was 0.86, BI was 1.31 and PC was 2.22 units. These experimental values were very close to the predicted values, suggesting the suitableness of the models developed in the current study.

### 3.3. VAE and CE of anthocyanins from black carrots

The effectiveness of VAE and CE in producing high anthocyanin-rich extracts was evaluated. The crucial step for enhancing the release of phenolics and anthocyanins from black carrots is the disintegration of the cell wall polysaccharides which opens the cell and removes the physical barrier. Value of TAC obtained by VAE was 1375 mg/L. The anthocyanins output from black carrots using standard methods such as citric acid-acidified water was 323 mg/L, acetic acid-acidified water was 255 mg/mL, and acetic acid + sulphur dioxide at pH 3 was 444 mg/L. A comparison between the anthocyanins recovered by CE and VAE is given in Table 3.

### 3.4. Qualitative analysis of anthocyanins in black carrots extract

The anthocyanins extract was profiled using HPLC. The optimized extract of black carrots contained a combination of five anthocyanins classified on the basis of their UV spectra and literature data. These anthocyanins were cyanidin 3-coumarylxylosylglucosylgalactoside (CCG), cyanidin 3- xylosylglucosylgalactoside (CXG), cyanidin 3-sinapoylxylosylglucosylgalactoside (CSG), cyanidin 3-feruloylxylosylgluco sylgalactoside (CFG), and cyanidin 3-xylosylgalactoside CXGal) (Fig. 2A). During HPLC analysis, the detector was set at 520 nm "Empower 2" software was used for the integration of the peak areas.

## 4. Discussion

### 4.1. VAE

Enzyme-assisted extraction is considered as a better option compared to conventional solvent extraction, microwave-assisted extraction and ultrasound-assisted extraction, because it can improve the phenolics yield from fruits and vegetables sources by 40% with better bioavailability and comparatively less degradation (Belwal et al., 2018; Kumar et al., 2020). In our study, significant variations ($p < 0.01$) in TAC were observed in response to time, temperature and EC with VAE. The maximum value of TAC obtained experimentally using Viscozyme was 1375 mg/L, which was near to the predicted value of 1380 mg/L. Our results corroborate with the findings of other researchers, who indicated that Viscozyme enhanced the anthocyanin yield from chokeberry pomace (Kitryte et al., 2017). A similar increase in anthocyanins yield (from 504 to 1005 mg/L) from black carrots using cellulolytic enzymes was also confirmed by previous studies (Khandare et al., 2011). In the study conducted by Kaur et al (2016), black carrots juice extracted via enzyme-assisted processing had a high juice yield (86.31%) and high total monomeric anthocyanins (1252 mg/L) content, thereby demonstrating that Viscozyme is a potential enzyme combination for enhancing juice yields and anthocyanins content from black carrots. Other studies have also reported an increase in anthocyanins yield from grape vines, black currants, purple corn and elderberries through pectinase enzyme (Puertolas et al., 2011). Cellulolytic enzymes like Cellucast, Pectinex® BE Colour and Vinozym® FCE G, increased the phenolics extraction yield from mulberry (230.58–272.16 mg/100 mL), black currant (900–2200 mg/kg) and grape mash (Landbo & Meyer, 2004; Kammerer et al., 2005; Arnous & Meyer, 2010; Dinkova et al., 2014; Tchabo et al., 2015). Cell wall components, hemi-cellulases, amylases, and Viscozyme (mixture of carbohydrases) has been efficiently utilized for the extraction of phenolics (Teles et al., 2021; Macedo et al., 2021). A study suggests that anthocyanins could be extracted at a higher efficiency from saffron tepals using Pectinex Ultra SP-L enzyme when compared to the ethanol-based extraction (Lotfi et al., 2015). Moreover, the anthocyanins extracted by enzymes from saffron tepals exhibited increased resistance against decomposition and browning and displayed greater values of colour (more chroma and lightness) as well as increased chemical stability (less polymerization and degradation) when compared to those extracted with ethanol. Furthermore, a greater content of a highly stable anthocyanidin, cyanindin 3,5-diglucoside was found in the anthocyanins extracted with enzymes compared to recovered only using an ethanolic solution. Phenolic and anthocyanins are potent bioactive compounds involved in the prevention of diseases related to oxidative and nitrosative stress such as cancer, neurological disorders cardiovascular diseases and hypertension. It is crucial to extract and optimize these compounds from the plant matrices (Kumar et al., 2020). Anthocyanins help in the attenuation of lipopolysaccharide-mediated inflammatory gene expression in human adipocytes, and also play a critical role in the inhibition of α-glucosidase activity and counter type-2-diabetes (Sinopoli et al., 2019; Zhang et al., 2020). Anthocyanins are primarily positioned within the vascular inclusions in the sub-epidermal and epidermal cell walls called anthocyanoplast bodies. The compactness of the polysaccharide matrix of cell wall affects the ease of anthocyanin extraction, implying that the cell wall serves as a defensive barrier against anthocyanin extraction. Cell wall carbohydrates primarily resist the mass transfer by hindering the permeation of the enzyme that can specifically cleave the structural phenol-protein-polysaccharide linkages and hinders the unobstructed movement of released anthocyanins or other phenolic compounds from both cytoplasm and vacuoles (free phenolics/anthocyanins) as well as from the cell walls (bound phenols) (Kocia et al., 2013). Phenolic compounds are the largest group of plant secondary metabolites, which

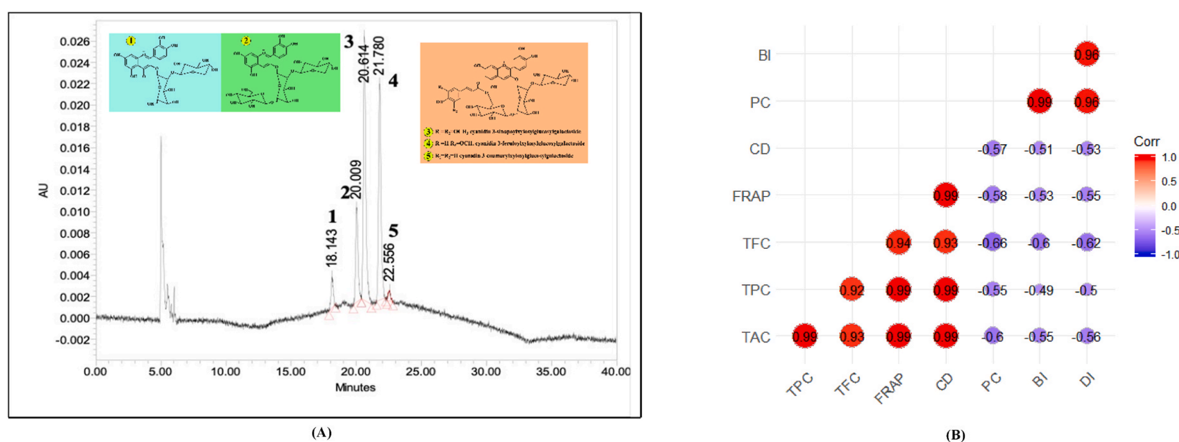


**Fig. 2.** **(A)** HPLC chromatogram of a black carrot extract. Where, (1) cyanidin 3-xylosylglucosylgalactoside (2) cyanidin 3-xylosylgalactoside (3) cyanidin 3-sinapoyl-xylosylglucosylgalactoside (4) cyanidin 3- feruloylxylosylglucosylgalactoside and (5) cyanidin 3- coumarylxylosylglucosylgalactoside. **(B)** Correlation matrix of bioactives, antioxidant activity and browning parameters. Where, TPC: total phenolics content; TFC: total flavonoid content; Antioxidant activity by FRAP: ferric reducing ability of plasma; CD: colour density; PC: polymeric colour; BI: browning index; DI: degradation index.

range in size from a simple structure with a phenol ring with low molecular weight to very complex structures like lignin and tannins with high molecular weight. Phenolics can be made up of a single phenolic acid or a group of phenolic acids (polyphenols), all of which have phenol rings with hydroxy functional groups; in their structures, those contribute to their antioxidant effects. These phenolics consist of phenolic acids, flavonoids, coumarins, isoflavonoids, stilbenes, lignans, and phenolic polymers (Kumar et al., 2020). Disintegration of the cell wall can markedly increase the release of phenolics, by curtailing hindrance. Enzymes with specific hydrolytic activities can be employed for disrupting the cell wall matrix to increase the accessibility to bioactive compounds like anthocyanins (Garcia, 2018; Kanha et al., 2019). Cellulase hydrolyzes the glycosidic bonds inside or at the ends of cellulose microfibrils. It catalyzes three types of reactions: first, it fragments the amorphous regions of cellulose microfibrils by hydrolyzing β-1,4 glucosidic bonds through endoglucanase, creating cellobiose. This cellobiose is further degraded by cellobiohydrolase (exoglucanase) to yield glucose and cellobiose. β-glucosidases then hydrolyze the cellobiases into glucose subunits (Gligor et al., 2019). Hemicellulose is a group of carbohydrate polymers that is weak, amorphous and serves as an important structural component of the plant cell wall. Typical compounds belonging to this group include β-glucans, xylans, arabinoxylans, and galactomannans. The synergistic actions of xylanase hydrolyzing xylans, hemicellulose hydrolyzing general hemicellulose, β-glucanase hydrolyzing β-glucans, and arabanase hydrolyzing arabanans present in Viscozyme catalyze a complete deconstruction of the cell wall in a cost-effective manner (Danalache et al., 2018; Garcia, 2018). The disintegration of cell wall using an optimum enzyme concentration along with increased temperature assist in the liberation of bound water molecules and the concurrent release of hydrophilic anthocyanins (Kocia et al., 2013). A detailed molecular mechanism of Viscozyme-based anthocyanins extraction is presented as a graphical illustration in Fig. 3. Table 2 clearly indicates a consequential effect of the interaction between reaction time and temperature on the total phytochemical extract, degradation parameters, CD and AOC ($p < 0.01$). The minus prefix of the factor indicates that the interaction between the factors resulted in a negative effect on the response, suggesting that sufficiently high temperature for a longer time reduces the total polyphenols concentration of the extract. Viscozyme concentration also affected the TPC ($p < 0.05$). The enzyme concentration had a positive effect on TPC for brief time intervals (up to 60 min), after which it starts to display a negative effect such as for an interval like 90 min. The results revealed that the variables were significant, and the quadratic model had a strong correlation. It was found accurate and acceptable for predicting achieving the maximum TPC, TFC, and TAC with high AOC and low anthocyanins degradation. Temperature plays a decisive role by disrupting the cell wall and unlocking the plant matrices, thereby permitting enzyme penetration and facilitating the release of phenolics (Costoya et al., 2010; Puri et al., 2012; Kanha et al., 2019). High temperature and longer maceration time increase the release of anthocyanins from grape vines (Hermosin et al., 2005). The incubation period is another decisive factor concerning VAE. Longer extractions are deleterious for the isolation of phenolic bioactives like anthocyanins that can easily degrade and polymerize due to their thermo-labile nature (Silva et al., 2007). In the study conducted by Kaur et al. (2016), the changes in Viscozyme concentration, temperature and incubation time had a significant effect on the juice yield and anthocyanins content of black carrots. With an increase in enzyme concentration and temperature, the juice yield and anthocyanins increased sharply but reached a plateau when extraction was conducted beyond 60 min. Under these optimized conditions, the experimental maximum juice yield and anthocyanins concentration were 86.3% and 1252 mg/L, respectively. There was excellent agreement between the experimental values and the predicted values, thereby indicating the suitability of the developed models and the success of RSM in optimizing extraction conditions (Kaur et al., 2016; Kumar et al., 2021). At optimum time duration is also needed for efficient and complete extraction of anthocyanins from the cellular matrix; otherwise, else they could stay entrapped and could go to waste in the pomace. Reports confirm that short extraction durations favour high phenolics extraction yields than longer extraction periods due to the autoxidation of phenolics (Ekici et al., 2015; Kanha et al., 2019). In the present investigation, the highest values for TPC and TAC were obtained at 60 min. Increasing the maceration time enhances the concentration of various anthocyanins in grape wines (Hermosin et al., 2005). Longer incubation periods stimulate the tertiary activity of Viscozyme, which causes the conversion of anthocyanins to anthocyanindins and the release of glucose. Longer extraction time can result in the cleavage and oxidation of covalent bonds in anthocyanins, causing their degradation (Tchabo et al., 2015; Tiwari et al., 2010). The effect of concentration and extraction time of a commercially available enzyme mixture, Pectinex®, was studied on the anthocyanins yield and colour properties from saffron tepals. An extraction period of over 3 h with more than 5% EC, resulted in negative extraction yields, substantiated by the aforementioned explanations. The degradation of anthocyanins is associated with an increase in the polymeric colour index, caused by the condensation reactions and melanoidin formation (Lotfi et al., 2015).

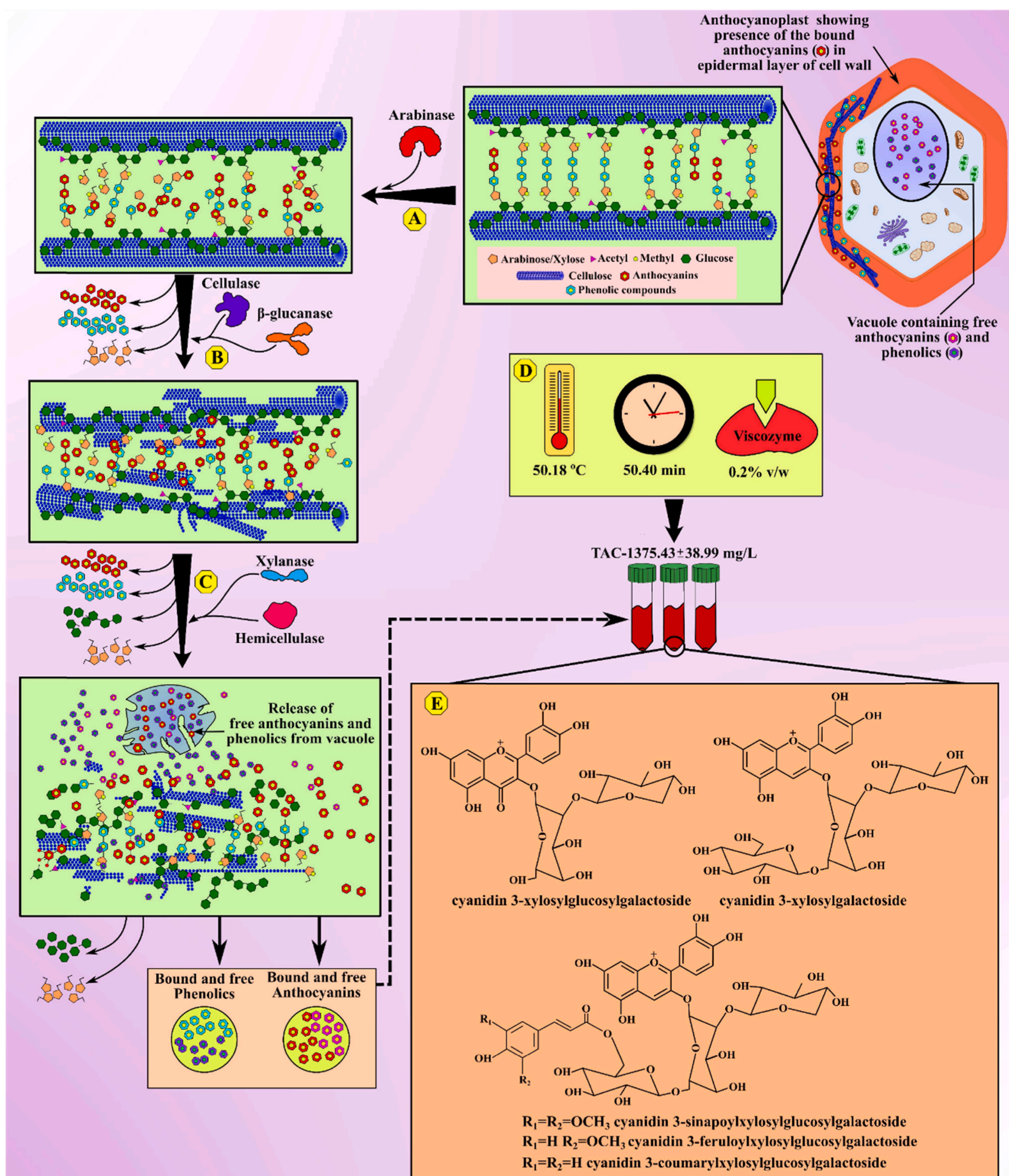


**Fig. 3.** Viscozyme-assisted extraction of anthocyanins and other phenolics from black carrots. A) Arabinase enzyme catalyzes the hydrolysis of arabinans present in the cell wall. This helps liberate bound phenolic compounds or anthocyanins present in between the cellulosic microfibrils in the cell wall of black carrots. B) Cellulase acts on the cellulose microfibrils present in the cell wall of black carrots and accelerates the breakdown of cellulose into glucose and cellobiose by acting on the glycosidic bond joining to the monomeric units in cellulose. β-Glucanase also acts in a similar manner and releases a glucose residue by cleaving the β-1,3 linkage between the glucose units in cell wall components. This helps in the loosening of the main structural component of the cell, solubilizing anthocyanins and other phenolics in the solvent system. C) The hemicellulase and xylanase disintegrate the β (1,4)-linkages between the monomeric units of cellulose and hemicellulose chain (xylan) as a result further loosening of the cell wall. This facilitates step helps in further release of phenolic compounds from the matrix of the cell wall. Hence, Viscozyme treatment (A+B+C) acts simultaneously and causes the disruption of bonds between phenolic compounds and cell wall constituent's components of black carrots and improves the extraction efficiency by releasing both free and bound phenolics. D) Optimized condition and yield of anthocyanins. E) Anthocyanin profile of an optimized black carrots extract.

*4.2. Functional characterization*

Anthocyanins are pigments with appealing hues that are currently being used as sources of natural colorants (Paulsmeyer & Juvik, 2021). The extract was characterized on the basis of function in terms of DI, BI, PC, CD and AOC, and various anthocyanins present in the extract were recognized by HPLC analysis. PC estimates the browning and the degree of anthocyanin polymerization. To estimate PC, anthocyanins are reacted with bisulphite to form a sulphonic acid adduct which is colourless, while the coloured polymerized anthocyanin–tannin complex resists bisulphite bleaching (Turkyılmaz et al., 2012; Rawdkuen et al., 2020). Lower values of DI, BI and PC indicate increased stability of anthocyanins and a decreased degradation rate (Turkyılmaz et al., 2012). More anthocyanins displayed a higher CD value of 67 units. DI, BI and PC values were 0.86, 1.31 and 2.22 units, respectively. The reaction between CTs like epicatechin or catechin and monomeric anthocyanins causes the formation of polymeric pigments. Diverse hydroxy residues from phenols in CTs interact with anthocyanins to form a chemical complex (Turkyılmaz & Ozkan, 2014; Dini et al., 2020; Türker & Doğan, 2021). Polyphenol oxidase causes an increase in the formation of PC with greater extraction temperature. This could be due to the formation of a chemical intermediate in the anthocyanin degradation pathway called chalcone (Tiwari et al., 2010; Wang et al., 2010). Similar variation in PC with respect to temperature was observed in blueberry juice (Michalska & Lysiak, 2015). BI signifies the change in colour from reddish to yellowish or brownish conferred by PC.

*4.2.1. Characterization of anthocyanins*

Even though black carrots are reported as being an ideal source of natural anthocyanins along with their acylated forms, there is no information on the composition of anthocyanins extracted from the black carrot variety (*Pusa asita*). The greater amount of phenolics and associated antioxidant activity in black carrots imparts free-radical scavenging activity; consequently, protecting the human body from various illnesses associated with degenerative diseases, carcinogenesis, and atherosclerosis. These antioxidant-rich phenolic extracts can further be used in nutraceuticals and the functional food industry (Kumar et al., 2020). Procyanidins were predominantly detected with sugar attachments that were non-acylated in case of compound 1 and 2, or acylated with sinapic acid (compound 3), ferulic acid (compound 4) or *p*-coumaric acid (compound 5) (Fig. 2A). A mixture of five different procyanidins was characterized from an optimized black carrot extract including (1) CXG (2) CXGal (3) CSG (4) CFG and (5) CCG (Fig. 2A) based on their UV spectra and literature data. The results are in agreement with previous reports (Kammerer et al., 2004; Algarra et al., 2014; Gras et al., 2016; Kaur et al., 2016). Researchers have also reported additional peaks, but these were absent in our HPLC profiles (Algarra et al., 2014). This could be attributed to a difference in environmental conditions and variety used.

*4.3. Correlation among bioactives, AOC and browning parameters*

Correlation matrix among different parameters revealed that DI was highly proportional to PC and BI and less proportional to CD, FRAP, TFC, TAC and TPC (Fig. 2B). Similarly, CD was inversely proportional to PC, BI and DI and highly proportional to TAC, TFC, TPC and FRAP.

## 5. Conclusion

Anthocyanins have been widely used in industries, including food, cosmetics, and pharmaceuticals due to their natural colorant, antioxidant and therapeutic properties. Viscozyme-assisted extraction was optimized to recover the maximum yield of water-soluble bioactive compounds from black carrots. Under the optimal conditions (enzyme/substrate: 0.2% v/w, 50 °C, 58.4 min) for VAE, there was an increase in the recovery of total soluble constituents and anthocyanins yield with minimum browning parameters. The multi-catalytic activity of Viscozyme on cell wall components of black carrots resulted in efficient extraction of bioactives with potent antioxidant activity. This extraction technique can certainly be used as an efficient, eco-friendly and cost-effective strategy for the extraction of anthocyanins from black carrots and other fruits and vegetables in food processing industries, being capable of preventing these natural colours from thermal damages and enzymatic degradation.

## CRediT authorship contribution statement

**Manoj Kumar:** Investigation, Writing – original draft. **Anil Dahuja:** Conceptualization, Methodology, Writing – original draft, Supervision. **Archana Sachdev:** Conceptualization, Methodology, Supervision. **Maharishi Tomar:** Writing – review & editing, Formal analysis, Software. **José M. Lorenzo:** Writing – review & editing, Visualization. **Sangram Dhumal:** Formal analysis, Software, Writing – review & editing. **Radha:** Formal analysis, Software, Writing – review & editing. **Deepak Chandran:** Writing – review & editing, Visualization. **Eldho Varghese:** Writing – original draft, Formal analysis, Software. **Supradip Saha:** Writing – original draft, Formal analysis, Software, Supervision. **K.V.S.S. Sairam:** Formal analysis, Software, Supervision. **Surinder Singh:** Formal analysis, Software, Writing – review & editing. **Marisennayya. Senapathy:** Formal analysis, Software, Writing – review & editing. **Ryszard Amarowicz:** Writing – review & editing, Formal analysis, Software. **Charanjit Kaur:** Conceptualization, Methodology, Supervision, Writing – original draft. **John F. Kennedy:** Writing – review & editing, Formal analysis, Software. **Mohamed Mekhemar:** Writing – review & editing, Formal analysis, Software.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113528.Where, TAC = total anthocyanin content, TFC = total flavonoid content, TPC = total phenolic content, AOC = antioxidant capacity, CD = colour density, PC = polymeric colour, BI = browning index, DI = degradation index.

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