ABSTRACT

**Background:** The kokum rind consists of high amount of anthocyanin. The traditional methods are not capable to extract anthocyanin completely.

**Aims:** The present study was focused on the optimization of enzyme pretreatment for extraction of anthocyanin from Kokum rind.

**Methodology:** A central composite design was used to optimize the enzyme-assisted extraction of anthocyanin from the Kokum rinds. Kokum rinds was pretreated with food-grade pectinase and cellulase enzymes separately and then subjected to acidified ethanol extraction. The factors investigated included enzyme concentration (0 – 2%), Incubation time (1-5 h) and pretreatment temperature (15–55°C).

**Results:** Overall, 107.17- to 63.71 - fold increase in anthocyanin recovery when treated with pectinase and cellulase, respectively was observed compared to the untreated rinds. From a response surface analysis of the data, a second-degree polynomial equation was developed which provided the following optimal extraction conditions i.e. enzyme concentration 0.5%, Incubation time 2.0h and temperature 35°C for pectinase pretreatment. Whereas, in case of cellulase the
optimum conditions was enzyme concentration 1.42%, Incubation time 4.0h and temperature 32°C. The obtained results strongly support the idea of using cell-wall degrading enzymes as an effective means for recovering anthocyanin from Kokum rinds.

**Keywords:** Kokum; anthocyanin; pectinase; cellulase; RSM.

**1. INTRODUCTION**

The Kokum fruit is dark red in colour and consist of mainly three components i.e. Pulp/Juice, rinds/peel and seeds. The fruits are commercially processed for Kokum butter, syrups, squash, Agal (Kokum Juice) and amsul (Kokum rinds) and wastage is generated during processing of these products. The anthocyanins consist of varied groups of intensely colored pigments. These imparts orange, red, purple, and blue colors of many fruits, vegetables, flowers, leaves, roots and other plant organs [1-4]. The fresh fruit kokum contain 2.4 g/100 g anthocyanins [5].

Anthocyanan has been reported for the treatment of dysentery, tumors, heart complaints, stomach acidity, and liver disorders [6-7]. Eye-catching shade and solubility in water allows anthocyanin incorporating in aqueous food systems as well as possible health benefits, which considered anthocyanin as potential replacement for synthetic colour [6-10]. The red color of kokum is ascribed to the presence of anthocyanins such as cyanidin-3-glucoside and cyanidin-3-sambubioside [5,11]. The anthocyanin has high potential as a natural food colorant due to its possible usage in the production of confectionery, jellies, jams, health beverages and squash-like products, red wine, and desserts [6-7].

The enzymes like cellulase and pectinase are commercially in food processing for clarification of juices, to improve juice yield and extraction of bioactive components. Many researchers are reported used of enzymes for the extraction of pigments from plant sources such as precarthamine from safflower florets [12]; carotenoid from marigold flowers [13]; Anthocyanin from red grapes [14]; lycopene from tomato [15,16]; anthocyanin from black current [17]; anthocyanin from mulberry [18], Bioactive components from sweet cherry [19].

The present investigation aims to optimize enzyme added extraction of anthocyanin from Kokum rinds using response surface methodology, hence improving its yield.

**2. MATERIALS AND METHODS**

**2.1 Chemicals and Enzymes**

Acetone and Hydrochloric acid were purchased from Local supplier. Cellulase and pectinase were procured from Danisco India Pvt. Ltd., Mumbai (India). The cellulase was declared with 1470 U/mL activity at 55ºC optimum temperature and 4.0 – 4.5 pH, whereas pectinase declared with 160 U/mL activity at 50ºC optimum temperature and 4.0 – 5.0 pH.

**2.2 Kokum Rinds**

The Kokum rinds was procured from Ajramar Food Pvt. Ltd., Sagmeshwar, Ratnagiri district (MS), India. The seeds were separated; the foreign matter was removed packed in polypropylene bags and stored at 15±5°C until up to farther used.

**2.3 Enzyme-Assisted Extraction of Anthocyanin**

The Kokum rinds were pretreated with enzyme i.e. cellulase and pectinase for anthocyanin extraction. The Kokum rinds (3 g) were added 10 mL of cellulase and pectinase enzyme solution, prepared by dissolving the commercial enzyme in citrate buffer of pH 4.5 and 5.0, respectively [20]. The reaction mixture was continuously stirred and after completion of Incubation time, the enzymes were deactivated by heating at 80°C for 5 min. afterwards, subjected to anthocyanin extraction by acidified ethanol.

**2.4 Anthocyanin Assay**

The total anthocyanin was determined by the procedure suggested by Abdel-Aal and Hucl [21]. A macerated Kokum rind sample (3 g) was weighed in a 50-mL centrifuge tube, and 24 mL of acidified ethanol (ethanol and HCl 1.0N, 85:15, v/v) was added. The solution was mixed and adjusted to pH 1 with 4N HCl. The resulting solution was shaken for 15 min, readjusted to pH 1 if necessary, and the solution was shaken for
an additional 15 min. The tube was centrifuged at 7500 RPM for 15 min, and the supernatant was poured into a 50-mL volumetric flask and made up to volume with acidified ethanol. Absorbance was measured at 535 nm against a reagent blank. Cyanidin 3-glucoside (Sigma-Aldrich, India) was used as a standard pigment. Total anthocyanin content per sample (mg/kg) was calculated as cyanidin 3-glucoside:

\[ C (mg/kg) = \frac{A \times \text{Volume made} \times \text{MW} \times 10^6}{e \times 1000 \times \text{wt of sample}} \]

Where,

\( A \) is absorbance reading, \( e \) is molar absorptivity (cyanidin 3-glucoside 25,965 cm\(^{-1}\) M\(^{-1}\)) [21], MW is molecular weight of cyanidin 3-glucoside = 449.

### 2.5 Experimental Design and Statistical Analysis

A central composite design (CCRD) [22] was used to study the effect of three independent variables i.e. enzyme concentration \((X_1)\), incubation time \((X_2)\) and pretreatment temperature \((X_3)\) on experimental response of anthocyanins yield. The statistical software package Design-Expert 8.0. The coded and actual values of independent variables are as mentioned in Table 1.

Overall, the experimental design consisted of total of 20 runs (8+6+6). The layout of experimental design of pectinase and cellulase pretreatment experiment obtained through the software and the experimental values of response are given in Tables 2 and 3, respectively.

Table 1. The coded and actual values of independent variables for enzymatic extraction of anthocyanins

| Independent Variables          | Code | Level in code form |
|-------------------------------|------|-------------------|
| Enzyme concentration (%)     | X1   | -2  0  1  2       |
| Incubation time (h)           | X2   | 1  2  3  4  5     |
| Pretreatment temperature (°C) | X3   | 15  25 35 45 55   |

Table 2. The layout of experimental design in terms of coded values for pectinase

| Std. Order* | Run Order** | X1 | X2 | X3 | Yield (%)* |
|-------------|-------------|----|----|----|------------|
| 1           | 16          | -1 | -1 | -1 | 1.4524     |
| 2           | 11          | -1 | 1  | -1 | 1.5874     |
| 3           | 19          | 1  | 1  | -1 | 1.5223     |
| 4           | 3           | 1  | 1  | 1  | 2.0325     |
| 5           | 9           | -1 | -1 | 1  | 1.4652     |
| 6           | 7           | 1  | 1  | -1 | 1.5912     |
| 7           | 6           | -1 | 1  | 1  | 1.5347     |
| 8           | 5           | 1  | 1  | 1  | 2.0354     |
| 9           | 18          | -2 | 0  | 0  | 1.1807     |
| 10          | 8           | 2  | 0  | 0  | 1.7348     |
| 11          | 17          | 0  | -2 | 0  | 1.5046     |
| 12          | 13          | 0  | 2  | 0  | 2.0142     |
| 13          | 15          | 0  | 0  | -2 | 1.6522     |
| 14          | 12          | 0  | 0  | 2  | 1.6701     |
| 15          | 14          | 0  | 0  | 0  | 1.5784     |
| 16          | 20          | 0  | 0  | 0  | 1.5874     |
| 17          | 2           | 0  | 0  | 0  | 1.6012     |
| 18          | 1           | 0  | 0  | 0  | 1.5489     |
| 19          | 10          | 0  | 0  | 0  | 1.5645     |
| 20          | 4           | 0  | 0  | 0  | 1.5987     |

* The formal order of runs, ** Randomized order in which experiments are conducted; # Percentage yield of extracted Anthocyanins
3. RESULTS AND DISCUSSION

3.1 Effect of Pectinase and Cellulase Pretreatment on Extraction of Anthocyanins

The Kokum processing rinds was pretreated with pectinase and cellulase and its effect on the extraction yield of the anthocyanin was monitored and obtained results are presented in Tables 2 and 3, respectively. The untreated Kokum processing rinds samples showed 0.989 g/100 g yield of anthocyanin. Anthocyanin content of different varieties in the range of 1.90 to 2.35 g/100 g anthocyanin content [23]. The rind contains seeds and along with other foreign materials, which may be responsible for lower anthocyanin content. The anthocyanin yield was increased by 107.17- and 63.71- fold, when treated with pectinase and cellulase, respectively. The increase in enzyme concentration increases the yield of anthocyanins in both enzyme i.e. cellulase and pectinase treatments. The higher recovery of anthocyanins was obtained at 0.5% and 1.4% concentration of pectinase and cellulase enzymes, respectively. Low concentration of enzyme is able to open mainly the cells of the pulp, still attached to the peel, keeping most of the peel intact. Which would showed that the lower anthocyanins concentration of the collected chromoplasts.

Ladbo and Meyers [24] was tested five different processing enzymes those had pectinase, macerase, or protease activity, with different degrees of extraction recovery from black currant press cake residue. Enzyme added extractions resulted more polyphenolics, but were not observed to increase anthocyanins when judge against a control (no addition of enzyme). Particle size has shown to affect the quantity of phytochemicals extractable from pomace obtained from black currant, as the particle size reduced the amount extracted was increased. In addition, fine grinding was more influential than the use of commercial enzymes or heating in extraction of phytochemicals from blueberry juice–processing waste [25]. Cellulase and pectinase in phenolic extraction of grape pomace and reported that pectinase had optimistic effects; however cellulase had a negative effect in the extraction of polyphenolics [26].

The fruits peel consist of cellulose, hemicelluloses and pectin, which can be breakdown with the enzymes those have cellulolytic, hemicellulolytic and pectinolytic activities [27]. The yield of anthocyanin may be enhance when the cell-wall polysaccharides breakdown with the enzyme action. Plant cell-wall consist of cellulose, linear polymer of 1, 4 – linked glucose and hemicelluloses [28]. Bundles of cellulose molecules are aggregated together in the form of microfibrils composed of highly

### Table 3. The layout of experimental design in terms of coded values for cellulase

| Std. Order* | Run Order** | X1 | X2 | X3 | Yield (%)* |
|-------------|-------------|----|----|----|------------|
| 1           | 20          | -1 | -1 | -1 | 1.1336     |
| 2           | 11          | 1  | -1 | -1 | 1.2851     |
| 3           | 14          | -1 | 1  | -1 | 1.2954     |
| 4           | 18          | 1  | 1  | -1 | 1.5429     |
| 5           | 6           | -1 | -1 | 1  | 1.0188     |
| 6           | 12          | 1  | 1  | -1 | 1.3115     |
| 7           | 1           | -1 | 1  | 1  | 1.2653     |
| 8           | 17          | 1  | 1  | 1  | 1.3867     |
| 9           | 13          | -2 | 0  | 0  | 0.9931     |
| 10          | 9           | 2  | 0  | 0  | 1.4955     |
| 11          | 4           | 0  | -2 | 0  | 1.2114     |
| 12          | 16          | 0  | 2  | 0  | 1.5351     |
| 13          | 2           | 0  | 0  | -2 | 0.9878     |
| 14          | 5           | 0  | 0  | 2  | 0.9687     |
| 15          | 19          | 0  | 0  | 0  | 1.6014     |
| 16          | 15          | 0  | 0  | 0  | 1.5724     |
| 17          | 7           | 0  | 0  | 0  | 1.5124     |
| 18          | 3           | 0  | 0  | 0  | 1.5262     |
| 19          | 10          | 0  | 0  | 0  | 1.5561     |
| 20          | 8           | 0  | 0  | 0  | 1.5756     |

* The formal order of runs, ** Randomized order in which experiments are conducted; # Percentage yield of extracted Anthocyanins
ordered crystalline domains and extended amorphous regions.

3.2 Model Fitting

The central composite design was used to analyze the effect of process variables on anthocyanin extraction yields. The experimental results presented in Tables 2 and 3 were correlated by the following full 2nd-order polynomial equation:

\[ y = a_0 + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{3} a_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} a_{ij} x_i x_j \]  

(1)

Where, \( y \) is the Anthocyanin extraction yield, \( a_0 \) is the intercept, \( a_i, a_{ii} \) and \( a_{ij} \) are the linear, pure quadratic and interaction regression coefficients, respectively, and \( x_i \) are the coded independent variables.

With the help of stepwise regression method, with independent variables entered and eliminated at a confidence level of 90%, the following regression equation was established.

\[ y = a_0 + a_1 x_1 + a_2 x_2 + a_{12} x_{12} + a_{11} x_1^2 + a_{22} x_2^2 \]  

(2)

The estimates of the regression coefficients for pectinase and cellulase pretreatments obtained by the least squares method together with their standard errors, t statistics and corresponding p-values. Eq. 2 was found to be adequate to fit all experimental data with a coefficient of determination (R2) of 0.96 and 0.97, respectively.

Furthermore, in case of pectinase enzyme pretreatment, the mean sum of squares for pure error (MSPE) was (0.0004 with 5 degrees of freedom) was compared to the mean sum of squares for lack of fit (MSLoF = 0.002 with 9 degrees of freedom) of the above polynomial, their ratio (MSLoF /MSPE = 5.0) (Table 5a) was less than the percentage point of the F distribution (5.04) at the 95% confidence level. Whereas, in case of cellulase enzyme pretreatment, the mean sum of squares for pure error (MSPE = 0.0011 with 5 degrees of freedom) was compared to the mean sum of squares for lack of fit (MSLoF = 0.0021 with 8 degrees of freedom) of the above polynomial, their ratio (MSLoF /MSPE = 1.90) (Table 5b) was approx. equal to the percentage point of the F distribution (1.9) at the 95% confidence level. Thus indicating that lack of fit was not significant and that the simplified polynomial was a reasonable approximation to the mean structure in the region of experimentation. Therefore, the overall error variance of this composite design was estimated by combining the mean sum of squares for lack of fit and pure error as equal to 0.0024 with 14 degrees of freedom and 0.0032 with 13 degree of freedom for pectinase and cellulase pretreatments, respectively. Lastly, by referring to the last column in Table 4, it was possible to assess the significance of the individual regression coefficients as, that the linear coefficients \( a_0, a_1, a_2 \) and the quadratic coefficient \( a_{11} \) and \( a_{22} \) were significant at the 99% confidence level.

Table 4. Estimation of regression coefficients of Eq. 4 together with their corresponding standard error (s), t-value and p-value

| Coefficient | Pectinase | Cellulase |
|-------------|-----------|-----------|
| \( a_0 \)  | 1.614     | 1.560     |
| \( a_1 \)  | 0.149     | 0.110     |
| \( a_2 \)  | 0.128     | 0.087     |
| \( a_{11} \)| 0.094     | -0.079    |
| \( a_{22} \)| -0.032    | -0.047    |
| \( a_{33} \)| 0.043     | -0.150    |

Table 5a. Analysis of Variance for regression equation of pectinase pretreatment

| Source          | DF | SS   | MS    | F      | p-value |
|-----------------|----|------|-------|--------|---------|
| Regression      | 5  | 0.800| 0.1600| 107.10 | 0.000   |
| Residual Error  | 14 | 0.021| 0.0014| 5.04   | 0.0588  |
| Lack-of-fit     | 9  | 0.019| 0.0020| 5.04   | 0.0588  |
| Pure error      | 5  | 0.005| 0.0011|        |         |
| Total           | 19 | 0.820|       |        |         |
Table 5b. Analysis of Variance for regression equation cellulase pretreatment

| Source            | DF | SS    | MS   | F     | p-value |
|-------------------|----|-------|------|-------|---------|
| Regression        | 6  | 0.930 | 0.1500 | 87.3  | 0.000   |
| Residual Error    | 13 | 0.023 | 0.0017 |       |         |
| Lack-of-fit       | 8  | 0.018 | 0.0021 | 1.9   | 0.232   |
| Pure error        | 5  | 0.005 | 0.0011 |       |         |
| Total             | 19 | 0.95  |       |       |         |

3.3 Analysis of Response Surface

Response surface plot for the yield of anthocyanin by enzymatic (Pectinase and cellulase) extraction as a function of two independent variables while the other independent variable is set to their zero level is reported in Fig. 1 (a and b), respectively. Surface plot as a function of enzyme concentration (A) and Incubation time (B) show a plane-like dependence of anthocyanin yield on these variables. In fact, from Eq. 2 it can be seen that the effect of these variables on the response is described by linear terms. Consequently, whatever the values of the levels of the remaining variables, an increase of enzyme concentration or incubation time will result in an increased anthocyanin yield, the regression coefficient estimates of a1 and a2 being positive.

3.4 Model Validation

Several experiments were carried out, as per the optimal solutions to validate the model. The experiments were performed as per formulations given in Table 6. By using Eq. (2) together with the parameter estimates listed in Table 4, it was possible to calculate the predicted anthocyanin yield under the experimental conditions adopted for the validation experiments as well as its 95%-prediction interval (Tables 2 and 3). Furthermore, all the predicted responses were included into the 95%-prediction intervals.

Fig. 1. Response surface plot showing effect of (a) pectinase and (b) cellulase concentration and pretreatment time on extraction yield of anthocyanin when pretreatment time is held at its central value.
Table 6. Observed (y) and predicted (y) Anthocyanin extraction yields under different experimental conditions together with their 95% prediction intervals (PI) by the response surface model

| Sr. No. | X1   | X2   | X3   | Y(%) | Y_pred (%) | PI(%)  |
|---------|------|------|------|------|------------|--------|
| For Pectinase |      |      |      |      |            |        |
| 1       | 1.50 | 4.0  | 35   | 2.05 | 2.00       | 1.9 - 2.1 |
| 2       | 1.50 | 3.9  | 35   | 2.02 | 1.97       | 1.8 - 2.0 |
| 3       | 1.35 | 4.0  | 35   | 1.97 | 1.95       | 1.8 - 2.0 |
| For Cellulase |      |      |      |      |            |        |
| 4       | 1.40 | 4.0  | 32   | 1.60 | 1.62       | 1.5 - 1.7 |
| 5       | 1.10 | 4.0  | 36   | 1.62 | 1.61       | 1.5 - 1.7 |
| 6       | 1.50 | 3.5  | 30   | 1.62 | 1.60       | 1.5 - 1.7 |

4. CONCLUSION

The results of this study indicate that the recovery of anthocyanin from the Kokum rinds can be greatly enhanced by the use of pectinase and cellulase enzymes. In particular, pretreatment of Kokum rinds by pectinase and cellulase resulted in 107.17- to 63.71-fold increase in extraction yields. This fact, the comparatively low cost of commercial food-grade enzyme preparations lends strong support to the possible implementation of the process on an industrial scale. We have also shown that the response surface methodology is an appropriate method for determining the optimum values of the factors studied. At present, considerable amounts of Kokum processing rinds is produced as a solid waste. As a result, the exploitation of this material as a source of natural anthocyanin could not only provide economic benefits but also contribute to environmental protection.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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