Chapter 3

To standardize the microwave and ultrasound assisted extraction of pectin from Assam lemon peel and its physicochemical characterization

3.1. Introduction

Pectin is a natural component of plant cellular structure. It is composed of sugar molecules, primarily D-galacturonic acid, which are linked together by α-1,4 glycosidic bonds (Sun et al., 2023). Pectin gets partially esterified with methanol or acetic acid, which gives it its unique properties. It is recommended as a safe ingredient as an additive, without any limit on daily intake, by the Joint FAO/WHO committee. Pectin is utilized in value-added goods based on its structural and molecular attributes, including the DE value, galacturonic acid content, and molecular weight. Based on the DE value, pectin is categorised into high methoxy (DE>50%) and low methoxy pectin (DE<50%) (Flutto, 2003). Due to its complex hydrophilic nature, pectin finds application as a thickener (Islam et al., 2023), texture modifier (Kazemi et al., 2023), emulsifier (Firat et al., 2023), gelling, and coating agent in the food sector (Gurev et al., 2023; Zhou et al., 2023). Initially, it was used mainly to process jams, jellies, and marmalades (Akusu and Chibor, 2020). Recently, pectin has gained importance in food products such as bakery fillings (Ajibade and Ijabadeniyi, 2019), prebiotics and dairy products (Gomez et al., 2016; Yuliarti et al., 2019; Khubber et al., 2021), soft drinks, fruit beverages, confectionery, conserves and glazing (Chandel et al., 2022).

Bioactive products from natural sources can be effectively obtained for use in food systems through the process of extraction. However, it is a laborious process due to the high moisture content in raw materials like lemon peels or pomace, making them susceptible to fungal enzyme degradation. Fungal enzymes, including de-esterifying (pectin methylesterase) and depolymerizing enzymes (pectin lyase, polygalacturonase, and pectate lysase), play a key role in breaking down pectin (Chandel et al., 2022). Several extraction factors, such as particle size, pH, temperature, extraction duration, solvent type (Yeoh et al., 2008), and drying methods (Monsoor, 2005), significantly influence pectin yield. Smaller substrate particles yield more pectin as they contain more protopectin than larger particles (Ma et al., 2019). Earlier investigations have documented the extraction of pectin through UAE from various citrus species including *C. limetta* (Panwar et al., 2023), *C. sinensis* (Kamal et al., 2021), and *C. aurantium* (Hosseini et al., 2019). Similarly, previous studies on pectin extraction using MAE from multiple citrus species such as *C. limon* (Twinomuhwezi et al., 2023), *C. maxima* (Arora et al., 2023), and *C. limetta* (Sharma et al., 2023) has been reported. Chemical extraction methods are commonly employed for extraction of pectin from fruit. However, they pose significant environmental concerns due to the generation of acidic wastewater (Yang et al., 2018a). Therefore, novel extraction techniques such as UAE and MAE offer a cleaner, more efficient, and environmentally friendly approach. UAE reduces solvent usage, shortens extraction time, enhances repeatability, increases the purity of the obtained product, and consumes less energy compared to conventional extraction methods (Colodel et al., 2019). MAE has been developed as an alternative to traditional approaches. It boasts several advantages, including low energy consumption, easy control, short processing time, minimal solvent requirements, affordability, high efficiency, and cleanliness (Tongkham et al., 2017).

Presently, a number of studies have been published on the other varieties of *Citrus limon*; however, the Assam variety of *Citrus limon* needs further investigation due to limited scientific information. The process of intensification of conventional pectin extraction and changes in functional properties can be achieved by using microwaves, ultrasound, high pressure etc. The present objective is focussed to extract the pectin from Assam lemon peel using different extraction techniques such as CE, MAE and UAE and standardisation of pectin extraction conditions and their quality characterization using the DE, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM) analysis.

3.2. Materials and Methods

3.2.1. Raw materials and chemicals

The Assam variety of lemon, scientifically known as *Citrus limon* and commonly referred to as Assam lemon, was acquired freshly from the Citrus Garden, Tezpur University, Assam, India, during the period of June-July, 2021. The fresh lemons were washed under running tap water, after which they were peeled using a handheld peeler. Subsequently, the peels were dried at 45°C for 10 h within a laboratory-scale tray drier. Once dried, they were finely powdered using a domestic mixer grinder. The resulting finely ground powder was then sifted through a 40-mesh sieve and stored at ambient room temperature until needed for further applications. Ethanol (≥99%) was procured from a reputable retail source, while all other chemicals of analytical grade were purchased from HiMedia Laboratories Private Ltd. (Mumbai, India).

3.2.2. Experimental design

Pectin was extracted from Assam lemon by comparing three methods, namely conventional extraction (CE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). For each of the extraction processes, citric acid with a pH of 2 was selected as the extraction solvent. Citric acid is an organic acid with a low ability to break down pectin and has a lower hydrolysing potential than mineral acid; moreover, organic acid is better for the environment (Maric et al., 2018). Each of the extraction processes was modelled and optimized using the three-level, three-factorial Box-Behnken design (BBD), which resulted in 17 experimental runs and process parameters and their range was chosen based on the preliminary studies to study their effect on the response pectin yield (%). The general quadratic equation involving process variables was presented in equation Equation 3.1 (Altemimi et al., 2020).

$$Y = \beta_o + \sum_{i=1}^{n} . \beta_i x_i + \sum_{i=1}^{n-1} . \sum_{j=i+1}^{n} . \beta_{ij} x_i x_j + \sum_{i=1}^{n} . \beta_{ii} x_i^2 \tag{3.1}$$

Where β_o, β_i, β_{ii} and β_{ij} represent the coefficient of coded process parameters, and n represents process parameters considered in the study, i.e., 3. The above equation, Equation 3.1, can be depicted as a more contextual polynomial equation as expanded in equation Equation 3.2.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta_5 x_2 x_3 + \beta_6 x_3 x_1 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 \tag{3.2}$$

Where x_1, x_2 and x_3 represent the independent variables.

All the treatments were triplicated, and the effects of individual and interaction effects were analysed using regression analysis and ANOVA test using Design Expert (Version 12) software. The significance was assessed in terms of *F*-value at 95% confidence interval. The independent variables, including coded and actual for the experiments, are represented in Table 3.1. Each extraction method was optimized based on the maximisation of pectin yield.

3.2.3. Extraction of pectin

3.2.3.1. Conventional extraction (CE)

The independent variables selected for the conventional extraction of pectin from the peels of Assam lemon were temperature (X_{CT}), extraction time (X_{Ct}) and solid-liquid ratio (X_{CS}) with a range of 60 – 90°C, 20 – 100 min and 1:30 – 1:70 g/mL respectively, presented in Table 3.1. These parameters were used to study their effect on the yield of pectin. The dried peel powder was suspended in a beaker containing citric acid solution for the conventional extraction process, as per the solid-liquid ratio. Then the extraction process was carried out in a water bath (Model OVFU O-SRWB2, OVFU, Kolkata, India) that was kept at a predetermined temperature with continuous stirring and extracted for a specific time as per the experimental design. The resultant solution was filtered using muslin cloth, and the filtrate was collected and then subjected to purification (refer to section 3.2.4.).

3.2.3.2. Microwave-assisted extraction (MAE)

Microwave-assisted extraction of pectin from the peels of Assam lemon was performed using the microwave oven (Samsung-GE83ZL-N, Thailand). Microwave power (X_{MP}) in the range of 300 – 600 W, solid-liquid ratio (X_{MS}) range of 1:30 – 1:70, and extraction time (X_{Mt}) in the range of 3 – 7 min were selected as the independent variable for modelling of the microwave-assisted extraction process. Similar to CE, after the MAE extraction process, the mixture was filtered and purified to obtain pectin (refer to section 3.2.4.).

3.2.3.3. Ultrasound-assisted extraction (UAE)

In this method, the pectin from the peels of Assam lemon was extracted using a probe-type ultrasonicator (Model UW2070; Bandelin electronic, Germany) with 20 kHz. The effect of ultrasound amplitude (X_{UA}), solid-liquid ratio (X_{US}) and extraction time (X_{Ut}) on the pectin yield were studied. The range of each independent variable is shown in Table 3.1, along with coded and real values. The resultant solution was filtered using muslin cloth; the collected filtrate was subjected to purification (refer to section 3.2.4.).

3.2.4. Purification of pectin

The filtrate was obtained from the three-extraction methods CE. MAE and UAE consist of crude pectin, and the pectin was purified by the alcohol precipitation method (Guo et al., 2016). The purification process was carried out by precipitating filtrate with ethanol (99%) in a glass beaker with a 1:2 (v/v) ratio at room temperature for 3 h without stirring. Then, the precipitated pectin was centrifuged at 6500 rpm for 15 min, and wet pectin was separated. The separated wet pectin fraction was washed two times with ethanol, each time in a ratio of 1:1 to remove the monosaccharides and disaccharides. The obtained wet pectin was then dried in a hot air oven at 40°C until constant weight (Guo et al., 2016).

Table 3.1 Independent variables and their levels used in Box-Behnken Design

Extraction method	**Independent variable**	**Notation**	**Levels**		
			-1	**0**	**+1**
Conventional extraction (CE)	Temperature (°C)	X_{CT}	60	75	90
	Extraction time (min)	X_{Ct}	20	60	100
	Solid liquid ratio (g:mL)	X_{CS}	1:30	1:50	1:70
Microwave-assisted extraction (MAE)	Microwave power (W)	X_{MP}	300	450	600
	Extraction time (min)	X_{Mt}	3	5	7
	Solid liquid ratio (g:mL)	X_{MS}	1:30	1:50	1:70
Ultrasound-assisted extraction (UAE)	Ultrasound amplitude (%)	X_{UA}	20	60	100
	Extraction time (min)	X_{Ut}	15	30	45
	Solid liquid ratio (g:mL)	X_{US}	1:30	1:50	1:70

3.2.5. Physicochemical properties of pectin

3.2.5.1. Yield of pectin (%)

The yield of extraction is the quantity of extract retrieved in weight compared with the sample weight used for the extraction. The pectin yield was determined by the ratio of the weight of dried pectin to the weight of peel powder utilized for extraction under each extraction condition (Siddiqui et al., 2021), as mentioned in equation Equation 3.3.

$$Pectin\ yield\ (\%) = \frac{weight\ of\ dried\ pectin(g)}{weight\ of\ peel\ powder\ (g)} \times 100 \tag{3.3}$$

3.2.5.2. Determination of degree of esterification (DE)

Dried pectin (0.10 g) was moistened with 2 mL ethanol and then dissolved in 20 mL of distilled water at 40°C. As soon as the pectin/PIC was dissolved, five drops of phenolphthalein indicator were added to the conical flask, and the sample was titrated against 0.1 M sodium hydroxide solution. Upon colour change (colourless to pink), 10 mL of 0.5 M sodium hydroxide solution was added to the sample and mixed well for hydrolysis. After 20 min of room temperature incubation, 10 mL of 0.5 M hydrochloride solution was added to the solution and stirred until the pink colour completely disappeared. Finally, five drops of phenolphthalein reagent were added, and the solution was again titrated against 0.1 M sodium hydroxide until it turned a pale pink colour after vigorous shaking. The pectin's DE (%) value was determined using the formulae presented in Equation 3.4 (Hosseini et al., 2016).

$$Degree\ of\ esterification\ (\%)\ = \frac{V_2}{(V_1+V_2)} \times 100 \tag{3.4}$$

Where V_1 represents the volume of 0.1 M NaOH used in the initial titration step, and V_2 represents the volume of 0.1 M NaOH used in the final titration step.

3.2.6. Structural properties of pectin

3.2.6.1. Fourier transform infrared (FTIR) analysis

The dried pectin was mixed with potassium bromate (KBr) in the ratio of 1:10 w/w and hydraulic pressed to form a pellet. The pellet was then scanned in the FTIR spectrophotometer (Nicolet Instruments 410 FTIR, Thermo Scientific, USA) frequency range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1} (Fernández-Delgado et al., 2023) as recorded for pectin extracted from lemon peel powder.

3.2.6.2. X-ray diffraction (XRD) analysis

XRD analysis was performed to characterize the crystalline nature of the pectin samples. The crystalline diffraction patterns of pectin were recorded with XRD (Bruker AXS, Bruker D8 FOCUS, Germany) radiation kept at 40 kV and 40 mA, which was scanned at 2θ from 5° to 80° at a scanning rate of 4 deg min^{-1} (Mundlia et al., 2019).

3.2.6.3. Scanning electron microscopy (SEM)

The photomicrographs of the pectin were recorded at various magnifications (100x and 200x) after sputter coating with gold/palladium to investigate the surface morphology of pectin using SEM (JSM-6390LV, JEOL, Japan) operated at 20kV (Wang et al., 2020).

3.2.7. Statistical analysis

Data analyses were carried out using Design Expert (Version 12) and SPSS Statistics 17.0 (SPSS Inc, Chicago, IL, USA) software. The significance was assessed in terms of F-value at a 95% confidence interval. Analyses were performed on three replications, and averaged data for each experimental condition were expressed as mean ± standard deviation.

3.3. Results and Discussion

3.3.1. Modelling and optimization of CE of pectin from Assam lemon peel

The pectin yield (Y_{CE}) from the peels of Assam lemon during CE at different combinations of selected process parameters (X_{CT}, X_{Ct} and X_{CS}) varied between 8.37 to 20.18%. The Y_{CE} values obtained as per the experimental design were examined using the multi-regression method, particularly the consecutive model sum of squares, with linear, interactive, quadratic, and cubic models. Fitting the experimental data to a nonlinear regression equation presented in Equation 3.2 leads to the equation that can predict the Y_{CE} values. ANOVA was implemented to evaluate the acceptability and suitability of the developed model. The model F-value was found to be 26.02. From Table 3.2, the model was found to be statistically significant ($p \leq 0.05$), and the lack of fit was non-significant ($p > 0.05$). The diagnostic plot indicating the adequacy of model fit is indicated in Figure 3.1.

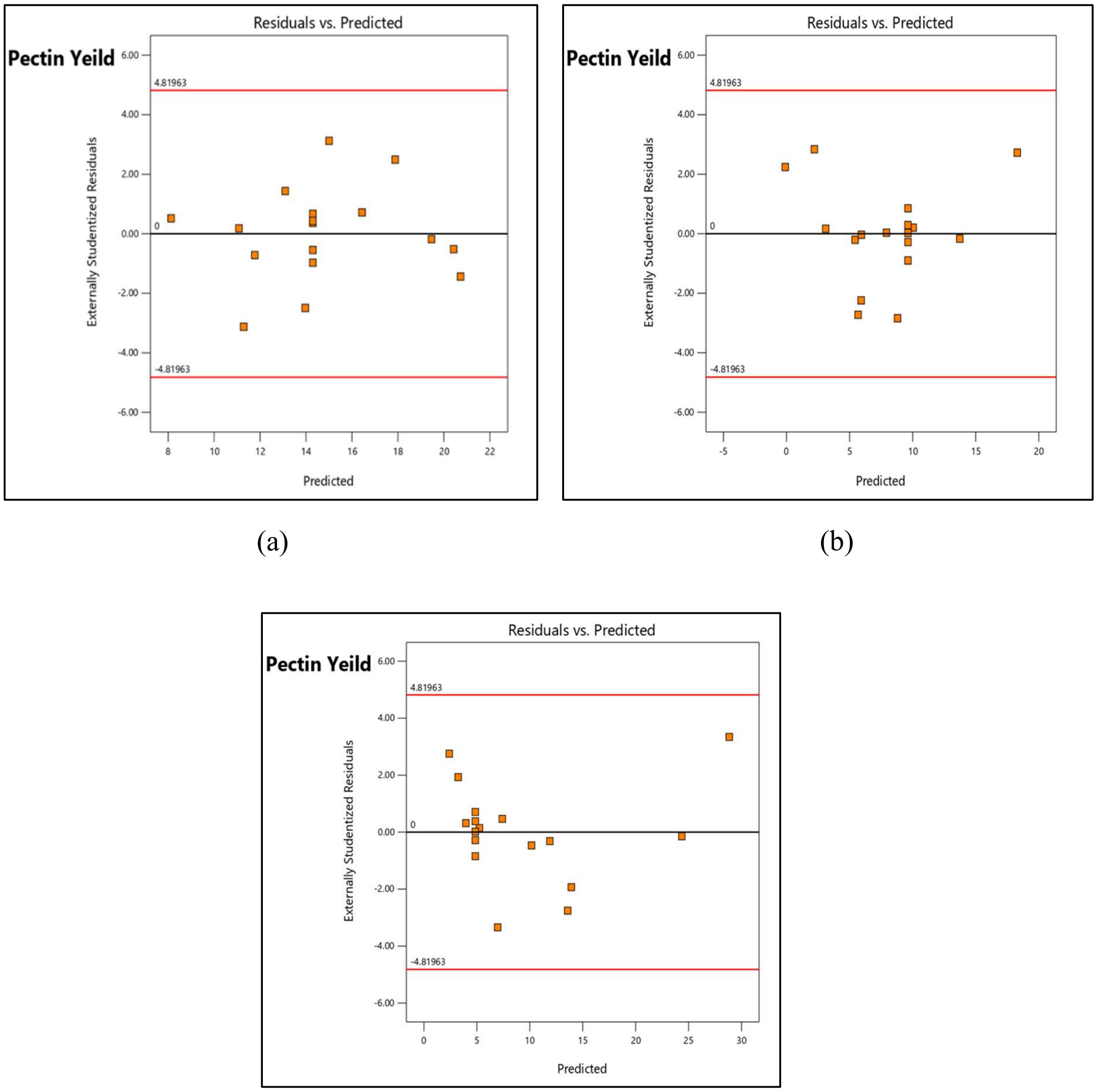
The model adequacy can be analysed through the statistical parameter correlation coefficient (R^2) and adjusted R^2. The R^2 and adjusted R^2 for the pectin yield obtained during the CE method were found to be 0.971 and 0.934, respectively, showing that these values did not differ significantly, and the non-significant terms were neglected from the model. The coefficient of variation implies the relative dispersion of the experimental data from the predictions of the evaluated model, and the lower value indicates that the deviations between experimental and predicted values were minimal (Maran et al., 2013). The coefficient of variation values was found to be 6.03%. "Adequate Precision" evaluates the signal-to-noise ratio, and a value higher than 4 is desirable, which indicates an adequate signal (Malviya, 2021). In the present study, the adequate precision was observed to be 18.461 for the pectin yield obtained during the conventional extraction method. Therefore, the model formed for Y_{CE} can predict the data with higher accuracy and the quadratic equation with significant parameters in coded form was presented in Equation 3.5.

$$Y_{CE} = 14.30 + 4.00x_{CT} + 2.14x_{Ct} + 1.55x_{CS^2} \tag{3.5}$$

The coefficient of each process parameter indicates their effect on the response. In Equation 3.5, the coefficient of temperature and extraction time was 4 and 2.14, respectively, which signifies the positive effect on the response Y_{CE}. This positive effect of an independent variable (x_{CT}, x_{Ct}, x_{CS^2}) on a dependent variable indicating an increase in the value of pectin yield. So, the increase in temperature or extraction time improves the pectin yield. The reason for the increase of pectin yield with an increase in temperature and time might be due to the higher solvent penetration into the solid matrix, causing the increase in the mass of polysaccharides to transfer from the solid particles into the solution (Charabaghi et al., 2017). The rise in temperature might have disrupted the cell wall of the plant material, which, in turn, allowed the quick separation and dissolution of pectin, resulting in a higher yield of pectin (Yu et al., 2021). Based on the magnitude of the coefficient, the temperature had a higher impact on the pectin extraction than the extraction time. Similar results were also reported during pectin extraction from passion fruit using the conventional extraction method, where the temperature and time had a positive influence on the yield of pectin and the temperature had a stronger influence on the yield of pectin than the extraction time (Dao et al., 2021).

There was a slightly negative effect of solid-liquid ratio (X_{CS}) on the pectin yield, as seen in Figure 3.2b and Figure 3.2c, which could be due to the absorption of energy by the excessive acidified medium (Rahmani et al., 2020). However, the effect of process variable X_{CS} on pectin yield was statistically not significant, as indicated by the ANOVA test ($p>0.05$), shown in Table 3.2. This effect might be due to the increased contact area between the extraction solvent and peel powder (Chua et al., 2020).

Another reason could be due to a higher driving force for pectin mass transfer induced by the higher concentration gradient (higher solid-liquid ratio), resulting in increasing the mass transfer rate of the pectin from the plant matrix to the extraction solvent (Chaharbaghi et al., 2017) according to Fick's first law of diffusion. The interaction effect of the independent variables was also statistically not significant (*p>0.05*).

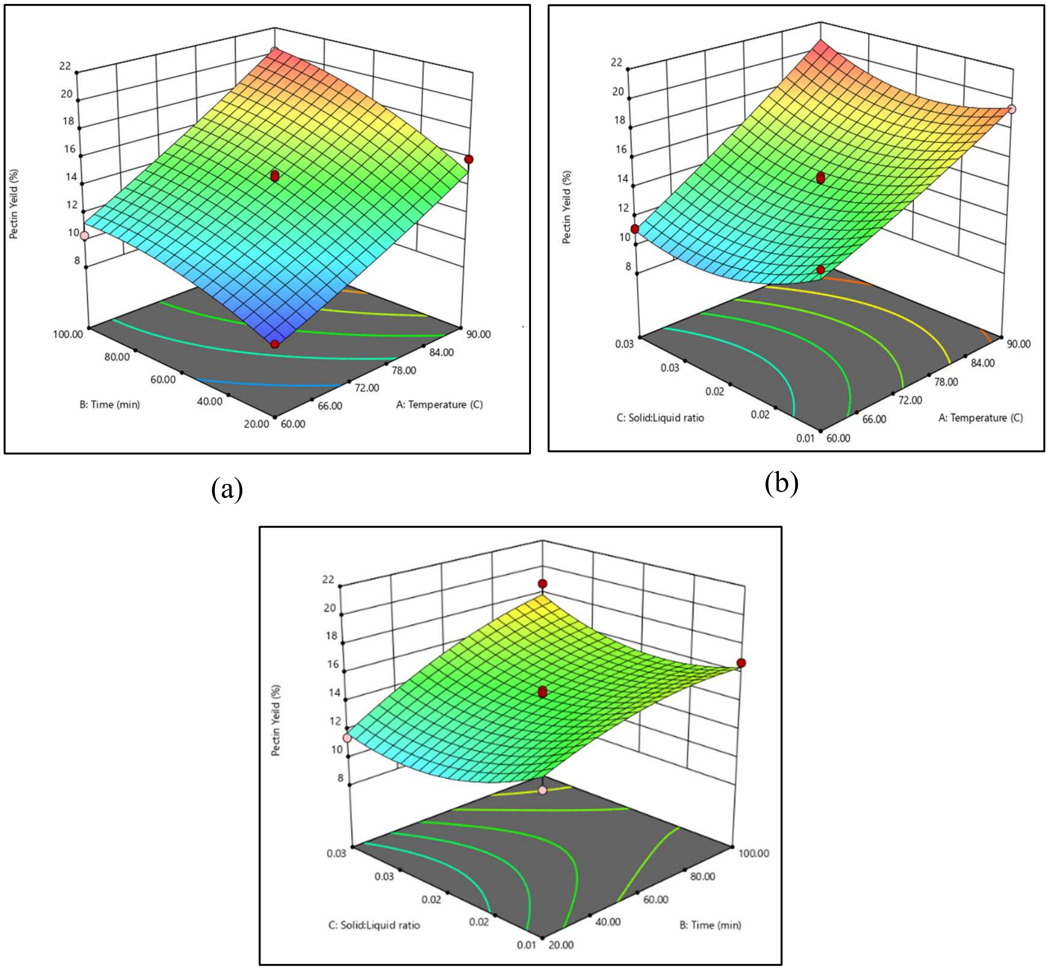


(a) (b)

(c)

Figure 3.1: Diagnostic plots of the model fit for pectin yield using (a) CE, (b) MAE and (c) UAE

The developed regression equation presented in Equation 3.5 was utilized to determine the optimum condition of process parameters for obtaining maximum pectin yield from Assam lemon peel. The regression model predicted the maximum pectin yield of 17.05% at a temperature of 90°C, extraction time of 22.69 min and solid-liquid ratio of 1:30 (g/mL) with 0.827 desirability. For validation purposes, the experiments were conducted at the optimum condition, and the pectin yield from the Assam lemon peel was observed to be 16.56 ± 0.24%. The relative deviation between the experimental and predicted values of the response pectin yield at the optimum condition was found to be 2.89%, exhibiting a good correlation between the values (Table 3.3).



(a)

(b)

(c)

Figure 3.2. Response surface plots of the extracted pectin through CE (a) interaction effect of time (min) and temperature (°C), (b) interaction effect of Solid:Liquid ratio and temperature (°C) and (c) interaction effect of Solid:Liquid ratio and time (min) on pectin yield (%)

3.3.2. Modelling and optimization of MAE of pectin from Assam lemon peel

MAE uses electromagnetic radiation in the microwave frequency range (300 MHz to 300 GHz) in the sample to spin and generate thermal energy in the solvent. Due to this effect, MAE is considered as an alternative method to conventional extraction as it reduces extraction time (Rodsamran and Sothornvit, 2019). Optimizing the extraction time was crucial in the case of pectin extraction by MAE method because the shorter extraction time may not produce the required heat to soften the carbohydrate polymer, and the longer extraction time can yield pectic acid by-product instead of pectin (Maran et al., 2015; Quoc et al., 2015). In the present study, the effect of MAE process variables, namely microwave power (X_{MP}), solid-liquid ratio (X_{MS}), and extraction time (X_{Mt}) was studied on the response pectin yield. The maximum and minimum pectin yield during the MAE method at different combinations of process parameters according to the BBD design was observed to be 20.60% and 1.99%, respectively. The maximum yield of pectin was obtained at 7 min of extraction time for the MAE method, whereas the CE method extracted the maximum pectin after 100 min of extraction time, which is 14 folds higher than the MAE extraction time. Comparable results were reported during pectin extraction from *Malus domestica 'Fălticeni'* pomace using the MAE and CE methods (Dranca et al., 2021). The formed quadratic equation for the pectin yield during the MAE method with significant process parameters in coded form was presented in Equation 3.6, and the ANOVA table related was presented in Table 3.2. From the Table 3.2, it was clear that the model was significant ($p \leq 0.05$) and lack of fit was not significant ($p > 0.05$). The diagnostic plot indicating the adequacy of model fit is indicated in Figure 3.1. The other statistical parameters R^2 was found to be 0.89, and the adequate precision was 10.20.

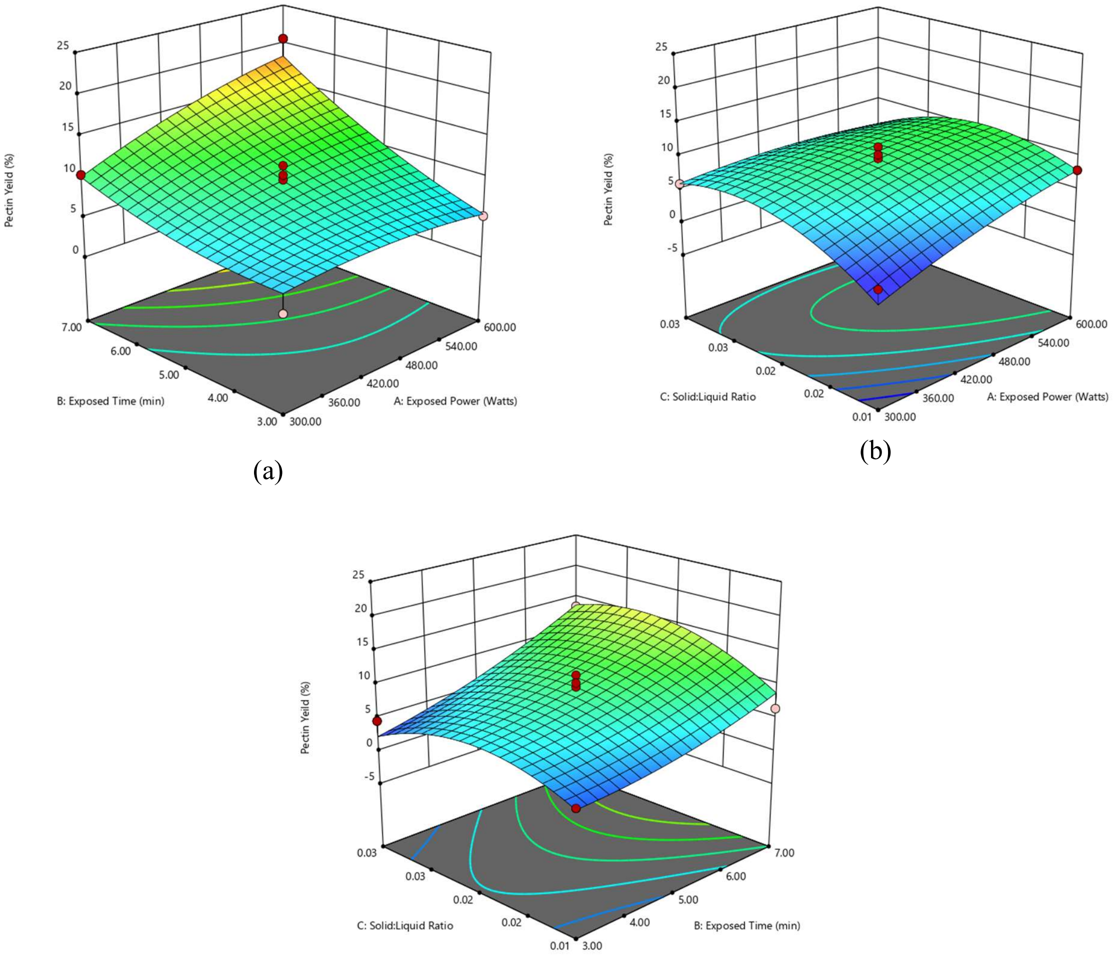
$$Y_{MAE} = 9.63 + 2.00x_{MP} + 4.31x_{Mt} - 3.80x_{MS}^2 \tag{3.6}$$

The regression Equation 3.6 obtained by non-linear regression analysis revealed that the microwave power and extraction time had a positive influence on the yield of pectin. The other process parameters, solid-to-liquid ratio, and the interaction terms were not significant ($p > 0.05$), as presented in Table 3.2. The time had most significant effect ($p \leq 0.05$) than the microwave power. With the increase of time from 3 min to 7 min at a particular X_{MP} of 600 W and X_{MS} of 1:50, the yield of pectin sharply increased from 5.09% to 20.61%. The higher yield of pectin at longer extraction times may be attributed to increased heat, which, in turn, softens cellulose and hemicelluloses by disrupting the hydrogen bonding in cellulose and hydrolysing glycosidic linkages in hemicellulose. This process leads to the breakdown of the branched structure that links pectin, causing a complete disruption of the cell wall matrix and facilitating the release of pectin (Liew et al., 2016a). A similar trend of increase in pectin from 10.48 to 17.97% was reported with an increase of extraction time from 1 to 3 min at a microwave power of 360 W using hydrochloric acid (mineral solvent) as a solvent during MAE of pectin from kiwi fruit peel (Karbuz and Tugrul, 2021).

The positive effect of microwave power on the pectin yield can be attributed to the improved penetration ability of solvent into the pectin source as the solvent temperature rises at higher microwave energy. This effect may help in rupturing the cell wall enabling the release of pectin from the plant material into the solvent (Zakaria et al., 2021). Lasunon and Sengkhamparn (2022) also reported that a rise of microwave power from 300 to 600 W increased the pectin yield at extraction times of 3 and 5 min during MAE of pectin from industrial tomato waste, but beyond the extraction time of 5 min, the yield was decreased with the increase of microwave power.

There was a slightly positive effect of solid liquid ratio (X_{MS}) on the pectin yield as seen in Figure 3.3. With the increase in the solid-liquid ratio, pectin yields increased due to the increase in the contact area between the peel and extraction solvent. However, after the solid-liquid ratio reached 1:70 (g/mL), pectin yield started degrading as most of the microwave energy was absorbed by the solvent instead of peel powder, resulting inthe lesser breakdown of the solid matrix and release of pectin from the solid matrix (Hosseini et al., 2016). However, the effect of process variable X_{MS} on pectin yield was statistically not significant as indicated by the ANOVA test ($p>0.05$), shown in Table 3.2. The interaction effect of the independent variables was also statistically not significant ($p>0.05$).

The optimum condition for MAE process parameters was found to be microwave power of 600 W, extraction time of 7 min and solid-liquid ratio of 1:30 (g/mL) to obtain a maximum pectin yield of 18.31% with desirability 0.876. At this optimum condition, the experimental pectin yield was found to be 19.61 ± 0.53%, and the relative deviation was 6.63%, which was less than 10%, which shows a good correlation (Table 3.3).



(a)

(b)

(c)

Figure 3.3. Response surface plots of the extracted pectin through MAE (a) interaction effect of exposed time (min) and exposed power (W), (b) interaction effect of Solid:Liquid ratio and exposed power (W) and (c) interaction effect of Solid:Liquid ratio and exposed time (min) on pectin yield (%)

3.3.3. Modelling and optimization of UAE of pectin from Assam lemon peel

The minimum pectin yield obtained from Assam lemon peel during the UAE method was observed to be 2.9%, and the maximum yield of pectin was 32.17%. When compared with the maximum yield obtained by the other two methods, CE (16.56 ± 0.24%) and MAE (19.61 ± 0.53%), at different combinations of process parameters, the higher pectin yield (32.17%) was obtained for the UAE process. The higher pectin extraction during the UAE method might be because of cavitation and thermal effect, leading to the disruption of the cell wall, allowing the less viscous medium to flow freely into the matrix and helping in accelerating, the release and diffusion of pectin (Chen et al., 2015b; Khedmat et al., 2020). The quadratic equation in coded independent variables obtained through nonlinear regression analysis was presented in Equation 3.7.

$$Y_{UAE} = 4.86 + 7.45x_{UA} + 3.49x_{Ut} + 4.99x_{UA}x_{Ut} + 3.11x_{UA}x_{US} + 5.68x_{UA}^2 \quad (3.7)$$

The model was significant ($p \leq 0.05$), and the lack of fit was not significant ($p > 0.05$), as presented in Table 3.2. The formed regression model was found to predict the data with higher accuracy, evidenced by the higher R^2 (0.954), adjusted R^2 (0.89) and the signal-to-noise ratio value (13.854). The diagnostic plot indicating the adequacy of model fit is indicated in Figure 3.1. The independent variables, such as amplitude and extraction time, are observed to have a positive influence on the pectin yield and can be visualized in Figure 3.4. The interaction terms $X_{UA}X_{Ut}$ and $X_{UA}X_{US}$ along with the quadratic term X_{UA}^2 was found to have a significant effect ($p \leq 0.05$) (Table 3.2). The interaction terms $X_{UA}X_{Ut}$ had a more significant effect on the yield due to its high F-value. This could be attributed to the synergistic effect of both ultrasonic intensity and time on cell wall disintegration and mass transfer efficiency. Out of the significant process parameters, ultrasound amplitude was found to have a higher impact on the extraction, followed by the extraction time. With an increase in the amplitude from 20 to 100 (%), the yield of pectin increased from 2.9 to 32.17%, as illustrated in Figure 3.4. At higher amplitude, the ultrasonication wave contains a high number of cycles of compression and rarefaction, resulting in the more violent collapse of bubbles. Thus, the targeted compound can rapidly be released into the solvent (Suhaimi et al., 2019). Comparable results of an increase in pectin yield with the rise of ultrasonication amplitude were reported during pectin extraction from sugar beet by the application of ultrasound (Jafarzadeh-Moghaddam et al., 2021).

The prolonged extraction time improved the yield of pectin in the solvent during the UAE method, which can be visualized in Figure 3.4. This phenomenon might be due to the fact that at the initial phase of extraction, the penetration of the solvent into the matrix was limited, and as the extraction proceeds, the intensity of the cavitation bubbles increased, resulting in effective permeation of solvent into the substrate, due to the increase of solvent temperature. Conversely, after reaching the saturation point, the extracted pectin might convert into a pectin by-product, pectic acid, resulting in a decrease in pectin yield (Maran and Priya, 2015). A similar trend of increase in pectin yield was reported with an increase of extraction time from 30 to 40 min during UAE of pectin from chayote (Ke et al., 2020).

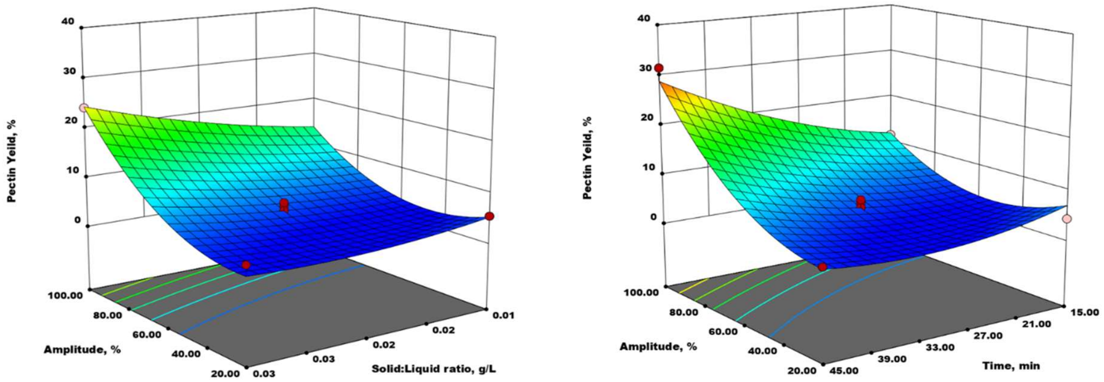
There was a slightly positive effect of solid liquid ratio (X_{US}) on the pectin yield. However, the effect of X_{US} on pectin yield was statistically not significant as indicated by the ANOVA test ($p>0.05$), shown in Table 3.2. This effect might be due to the fact that low volume of solvent results in higher consistency of the solution, which in turn reduces the rate of mass transfer and vice versa (Panwar et al., 2022; Pasandide et al., 2017). A comparable result was reported during pectin extraction from custard apple by the application of ultrasonication, where the increase of the solid-liquid ratio improved the pectin yield (Shivamathi et al., 2019). The interaction effect of $x_{US}x_{UT}$ was also statistically not significant ($p>0.05$).

The equation obtained in Equation 3.7 was used to optimize the UAE process parameters to obtain maximum pectin yield. The optimized condition for ultrasound amplitude, extraction time and the solid-liquid ratio was found to be 99.87%, 42.65 min and 1:30 g/mL, respectively, with a desirability of 0.98. Experiments were conducted in triplets at the optimum condition for validation and compared with the predicted value predicted by the response surface methodology (RSM). The experimental and predicted value for the optimum condition was found to be 32.17 ± 0.83% and 32.51%, respectively, with a relative deviation of 1.06% showing good agreement between them (Table 3.3). Moorthy et al. (2015) reported a 23.87% yield for pectin from pomegranate peel at a 1:17 g/mL solid-liquid ratio, 1.27 pH, 24 min, and 62°C. Lasunon and Sengkhamparn (2022) reported similar results of 32.77% pectin yield at 1.5 pH and a 1:20 solid-liquid ratio from industrial tomato waste. Similarly, Maran and Priya (2015) achieved a pectin yield of 29.32% from sisal waste under optimal ultrasonic conditions (ultrasonic power 61 W, 50 °C, 26 minutes, and 1:28 g/mL S/L ratio).

Table 3.2: The coefficients of polynomial equation and ANOVA test results indicating the effect of CE, MAE and UAE on pectin yield (%) from Assam lemon peel.

Coefficient	**CE**			**MAE**			**UAE**		
	Source	**Value**	***F*-value**	**Source**	**Value**	***F*-value**	**Source**	**Value**	***F*-value**
Intercept									
β_0		14.30			9.63			4.86	
β_1	x_{CT}	4.00	161.94*	x_{MP}	2.00	5.80*	x_{UA}	7.45	71.68*
β_2	x_{Ct}	2.14	46.49*	x_{Mt}	4.31	26.84*	x_{Ut}	3.49	15.70*
β_3	x_{CS}	-0.19	0.3564	x_{MS}	1.01	1.47	x_{US}	2.11	5.73
Interaction terms									
β_{12}	$x_{CT}x_{Ct}$	0.57	1.62	$x_{MP}x_{Mt}$	2.12	3.26	$x_{UA}x_{Ut}$	4.99	16.06*
β_{13}	$x_{CT}x_{\mathrm{CS}}$	0.82	3.41	$x_{MP}x_{\mathrm{MS}}$	-2.01	2.92	$x_{UA}x_{\mathrm{US}}$	3.11	6.25*
β_{14}	$x_{Ct}x_{\mathrm{CS}}$	0.91	4.19	$x_{Mt}x_{\mathrm{MS}}$	1.46	1.54	$x_{Ut}x_{\mathrm{US}}$	-0.40	0.10
Quadratic terms									
β_{11}	x_{CT}^2	0.25	0.33	x_{MP}^2	-0.90	0.62	x_{UA}^2	5.68	21.96*
β_{22}	x_{Ct}^2	-0.83	3.67	x_{Mt}^2	1.14	0.99	x_{Ut}^2	2.38	3.85
β_{33}	x_{CS}^2	1.55	12.75*	x_{MS}^2	-3.80	11.02*	x_{US}^2	1.15	0.90
Fit statistics									
Model (*F*-Value)		26.02*			6.04*			16.01*	
Lack of fit (*F*-Value)		4.12			5.23			6.23	
***R*²**		0.97			0.89			0.95	
Adjusted *R*²		0.93			0.74			0.89	
Adequate Precision		18.46			10.20			13.85	

*significa



(a) (b)

Figure 3.4. Response surface plots of the extracted pectin through UAE (a) interaction effect of Amplitude (%) and time (min) and (b) interaction effect of Amplitude (%) and Solid:Liquid ratio on pectin yield (%)

Table 3.3: Experimental and RSM predicted pectin yield values at optimum conditions for CE, MAE and UAE of pectin from Assam lemon peel powder

Extraction method	**Yield (%)**		**Relative deviation**
	Experimental	**Predicted**	**(%)**
CE	16.56±0.24	17.053	2.98
MAE	19.61±0.53	18.31	6.63
UAE	32.17±0.83	32.51	1.06

3.3.4. Characterization and comparison of pectin extracted in CE, MAE and UAE optimized conditions

3.3.4.1. DE of extracted pectin

The DE plays a crucial role in assessing the quality of pectin, as it reflects the carboxyl groups in galacturonic acid chains that undergo esterification with acetyl or methyl groups (Liew et al., 2016b). According to the experimental findings, Assam lemon peel-derived pectin exhibits a high methoxy content, exceeding 50% (Zoghi et al., 2021). Consequently, under optimal conditions, pectin extracted from Assam lemon peel can be utilized for its gelling properties and various food applications. The DE value of pectin obtained through

UAE (81.89 ± 3.27%) is significantly lower than that of MAE (84.28 ± 3.87%) and CE (90.29 ± 6.99%). This could be due to the prolonged extraction time in UAE, leading to the de-esterification of polygalacturonic chains in pectin from Assam lemon peels (Das and Arora, 2023). Additionally, it is observed that DE values do not correlate with the yield of pectin. It was observed that pectin extracted under high extraction temperatures and prolonged time led to a lower DE value; however, it achieved a higher yield.

3.3.4.2. FTIR analysis of extracted pectin

The FTIR spectrum of extracted pectin is mentioned in Figure 3.5. The absorption bands between 800 and 1200 cm^{-1} are known as the fingerprint region (Hosseini et al., 2016). The absorption peaks at 1010-1100 cm^{-1} signifies the presence of pyranoses and furanoses caused by the tensile vibration of C-OH, C-C and C-O-C (Kazemi et al., 2019; Qi et al., 2023). The peak at 827 cm^{-1} depicts the presence of α-D-mannopyranose.

The composition and structure of the extracted material can be influenced by the particular conditions applied during the extraction process. Varied extraction methods have the potential to change the molecular arrangement, resulting in differences in the FTIR absorption bands. The peaks at 1633 and 1440 cm^{-1} indicated carboxylate groups corresponding to the asymmetric and weaker symmetric stretching of the carboxylate group. The peak at 1738 cm^{-1} is due to the stretching vibration of the CO group of the carboxyl acid methyl ester (Wang et al., 2015b). There is an absorption band at 2935 cm^{-1} depicting a long chain linear aliphatic compound, attributed to C-H stretching vibration of the CH_3 associated with the O-acetyl groups (Trujillo-Ramirez et al., 2018). The absorption peak between 3650 and 3250 cm^{-1} depicts O-H stretching due to the intermolecular and intramolecular bonds, present in the main chain of galacturonic acid (Liang et al., 2012). Among the FTIR spectra of CE, MAE and UAE (Figure 3.5), there is a difference in the intensity of bands at 1738, 3650 and 3250 cm^{-1} that might be due to the pectin extracted from MAE and UAE exhibiting the depolymerization, de-esterification and probably deacetylation (Polanco-Lugo et al., 2019).

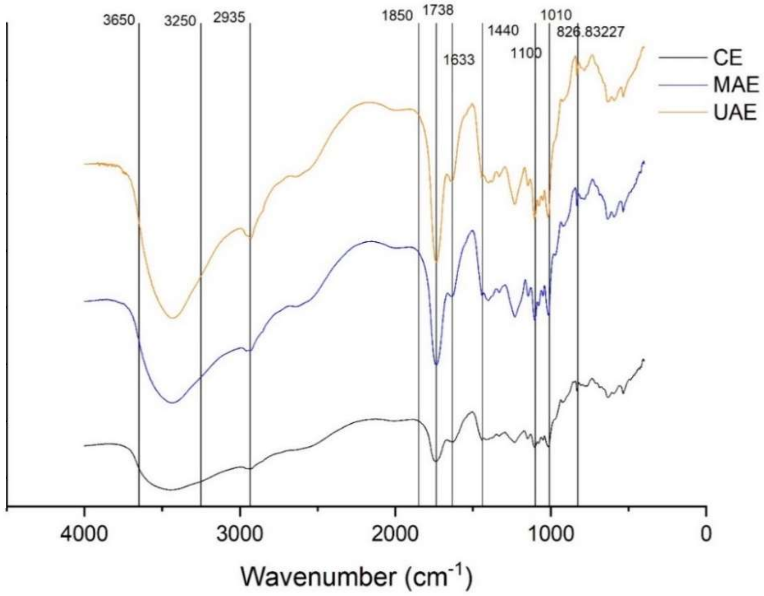


Figure 3.5 FTIR spectrum of extracted pectin through CE, MAE and UAE

3.3.4.3 XRD crystallinity analysis of extracted pectin

X-ray diffraction (XRD) is an important technique used to determine the crystalline or amorphous properties of pectin, which further helps in understanding its physical and functional attributes. The XRD graphs of extracted pectin through different extraction processes are mentioned in Figure 3.6.

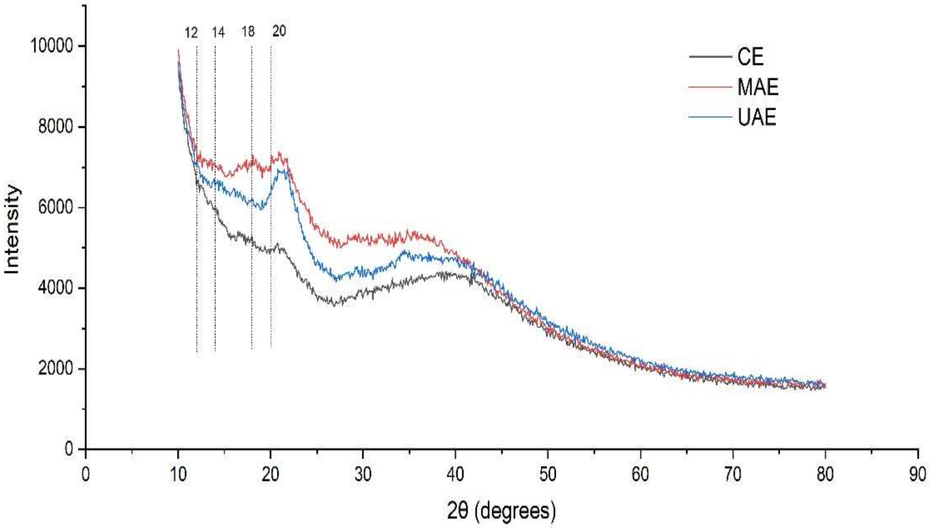


Figure 3.6. XRD spectrum of extracted pectin through CE, MAE and UAE

The XRD pattern of CE, MAE, and UAE pectin shows multiple broad diffraction bands, especially a broad and diffuse peak centred around 20° (2θ), resulting in a semi-crystalline to predominantly amorphous nature. This pattern is typical for polysaccharides like pectin, which have irregular molecular packing and limited crystalline domains (Perez et al., 2000). The sharp peaks at 12.50, 12.20, 13.50, 14.09, 15.59, 16.49, 16.09 and 17.99° (*2θ*) are due to crystallinity (Mundlia et al., 2019) in some portions of CE pectin. While in MAE pectin, the sharp peaks signifying crystalline portions are observed at 12.40, 12.70, 18.09, 18.39, 20.09, 20.89, 20.59 and 21.79° (*2θ*). In UAE pectin, the sharp peaks signifying crystalline portions observed are at 11.99, 12.60, 13.89, 14.29, 20.49, 20.79, 21.49 and 21.89° (*2θ*).

The pectin extracted from MAE exhibited the highest peak intensity at 20°, followed by UAE and CE. This indicates that MAE extracted pectin exhibited a higher degree of structural order or partial crystallinity, likely due to faster cell disruption and reduced degradation during extraction. The lower intensity and broader halo of CE extracted pectin (Figure 3.6) might be due to the prolonged heating in CE, which leads to the greater structural breakdown and higher amorphous content.

It can also be seen from Figure 3.6 that the UAE retained more structural order than MAE and CE, as mechanical cavitation in the UAE facilitates mild extraction conditions, thus exhibiting some semi-crystalline regions in the respective pectin. Similar results were seen by Panwar et al. (2023) stated that pectin extracted from *Citrus limetta* peels using UAE exhibited higher crystallinity.

3.3.4.4 Surface morphology of extracted pectin

The surface morphology of the extracted pectin under optimal conditions using multiple extraction techniques was examined by SEM, shown in Figure 3.7. The SEM micrograph of pectin exhibits relatively rough and compact flake structures. The SEM micrograph of pectin extracted using CE showed dense, irregular, and aggregated morphology, possibly due to prolonged acidic heating. This dense, aggregated morphology may result in lower solubility and slower gelling properties due to its tightly packed network (De Figueiredo et al., 2019). The microstructure of pectin extracted through MAE is more porous and fragmented. The morphology of MAE pectin seemed to be affected by a sudden increase in the high internal pressure and temperature caused by the MAE method (Kazemi et al., 2019; Dranca et al., 2020). This results in improved functional properties like solubility and gelling capacity. In the SEM micrographs of the UAE pectin sample, comparatively coarse morphology was obtained, as compared to that of CE and MAE pectin. While in SEM micrographs of UAE, the pectin samples seem to be less affected, which might be due to minimal thermal effect and cavitation resulting in rupture of the cell wall (Karbuz and Tugrul, 2021). The application of ultrasonic treatment resulted in a partially collapsed and exhibited a relatively coarse morphology in the microstructure of the extracted pectin (Yang et al., 2018a; Sengar et al., 2020). The SEM micrographs from Figure 3.7 indicated that extraction techniques such as MAE and UAE affected the morphology of the pectin.

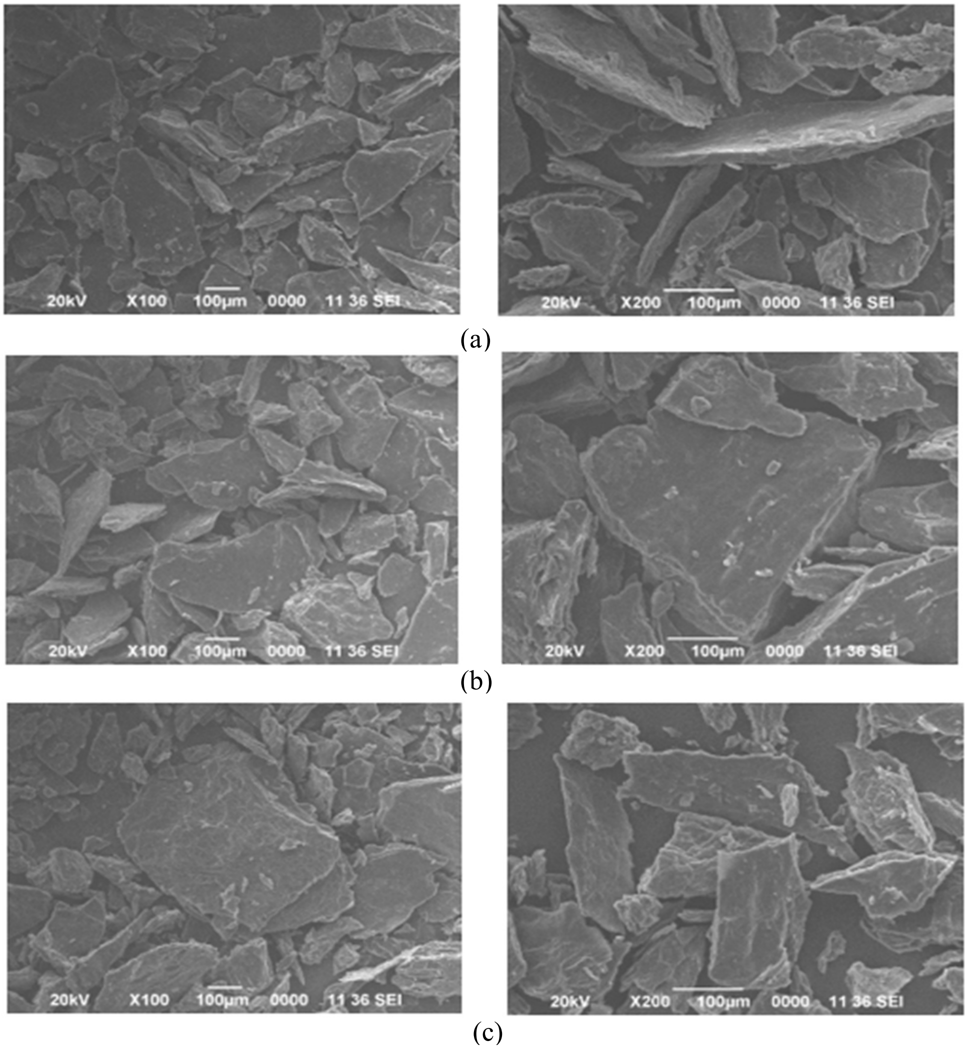


Figure 3.7. SEM micrographs of pectin (a) CE (b) MAE and (c) UAE