Optimization of Enzyme-assisted Revalorization of Sweet Lime (*Citrus limetta* Risso) Peel into Phenolic Antioxidants

Muhammad Mushtaq,[a,b] Bushra Sultana,[a,*] Haq Nawaz Bhatti,[a] and Muhammad Asgher [c]

Enzymatic treatment was used to induce liberation of antioxidant phenolics from under-utilized sweet lime peel (CLP). Small rotatable central composite design (SRCCD) was selected to optimize the conditions of enzymatic processing, *i.e*., enzyme concentration (2 to 5%), pH (5 to 8), temperature (30 to 75 °C), and incubation time (30 to 120 min). The morphological characterization of enzymatically hydrolyzed CLP was performed using field emission scanning electron microscopy (FESEM). Extracts obtained under suitable conditions were characterized for their phenolic profile by use of reverse-phase high-performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD) and evaluated for *in vitro* antioxidant activities. The observed results revealed that optimum enzymatic pre-treatment doubled the recovery of phenolic antioxidants with maximum Trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging potential (IC_{50}). The observed results indicate that enzyme-assisted extraction might be a green choice to revalorize agro-processing residues.

Keywords: Multi-response optimization; Citrus fruit; Antioxidant phenolics; TEAC; HPLC-DAD

Contact information: a: Department of Chemistry, University of Agriculture, Faisalabad-38040, Pakistan; b: Institute of Food Science, Cornell University, NY-14852, USA; c: Department of Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan;
** Corresponding author: bushrasultana2005@yahoo.com*

INTRODUCTION

Citrus limetta Risso belongs to the Rutaceae family, which encompasses about 40 species and is mostly cultivated in tropical and subtropical regions, with an annual production of more than 100 million metric tons worldwide (Moulehi *et al.* 2012). Citrus are one of the most extensively studied fruit crops because of their medicinal benefits against coronary diseases, chronic asthma, inflammation, tumors, and blood clotting (Alvarez *et al.* 1986; Bala and Grover 1989; Choi *et al.* 2002; Lagha-Benamrouche and Madani 2013). Epidemiological reports link the multiple medicinal properties of citrus to the presence of bioactive compounds such as phenolics, vitamin C, carotenoids, and flavonoids (Carmeli and Fogelman 2009; Abdillahi *et al.* 2011). In addition to the edible fruit parts of citrus, the non-edible parts, such as the peel, are also rich in bioactive compounds (Asikin *et al.* 2012; Asghar *et al.* 2013).

During postharvest processing, the peels from citrus fruit, which are half of the total volume, are mostly discarded. The current disposal of citrus peels presents not only an environmental issue, but also the loss of an opportunity to contribute to food security. Hence, attention has been focused on the revalorization of agro-processing wastes into valuable end use products.

The organized cell wall structure of citrus peel, which comprises pectin, a complex of heterogeneous polysaccharides, cellulose, hemicellulose, and lignins, is a barrier to the effective liberation of entrapped phenolics (Rimando and Perkins-Veazie 2005; Thielen *et al.* 2013). Phenolics in plant are bound to polysaccharide moieties through ether linkage (20-24 %) and cellulosic microfibrils via hydrophobic interactions and hydrogen bonding. These bound phenolics are further shielded by lignin and pectin materials. Solvents used in conventional solvent extraction (CSE) hardly distribute through compact cell wall structure and did not provide satisfactory extraction yield. The success of an extraction media or technique depends on soaking and distribution power of solvent system. In the present attempt, enzymatic pre-treatment was employed to improve cell rupturing and the mass transfer rates of aqueous methanol.

Enzymes can potentially hydrolyze pectin, cellulose, and glycosidic esters, and thus enhance the liberation of phenolic compounds entrapped covalently in the plant cell wall. However, enzymes are very specific in their activities and are decisively affected by experimental conditions, *e.g.*, temperature, pH, and substrate concentration (Bezerra *et al.* 2008). The current study presents an exhaustive and comprehensive economic optimization of enzymatic treatment parameters to revalorize citrus peel as an underutilized agricultural waste into nutraceutically and medicinally valuable phenolics with optimal level of retained antioxidant potential.

EXPERIMENTAL

Materials

Procurement of samples, materials, and chemicals

Healthy and mature sweet lime (*C. limetta*) samples were purchased from a local market in Faisalabad, Pakistan and further authenticated by the Department of Botany at the University of Agriculture, Faisalabad. The fruit peel was separated with a sharp knife, dried under ambient conditions (30 to 35 ˚C) for 5 to 7 days, ground into fine powder, and stored in air-tight polyethylene bags. Pectinex, celluclast, and alcalase enzymes, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 2,2-azinobis (3-ethylbenzothiazoline- 6-fulfonic acid) diammonium salt (ABTS), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, Folin-Ciocalteue reagent (FCR), myricetine, quercitin, as well as 3,4-dihydroxy benzoic, chromatotropic, ascorbic, *p*-hydroxy benzoic, gallic, linoleic, vanillic, caffeic, *p*-coumaric, ferulic, syringic, and sinapic acids were procured from Sigma-Aldrich (St. Louis, MO, USA). The chemicals ammonium thiocyanate, potassium persulfate, potassium ferrocyanide, Na_2CO_3, and acetic acid were purchased from Merck (Germany). Ultra-pure deionized water used for *in vitro* testing was obtained from Victor Lines Diagnostics.

Methods

Experimental design

Response surface methodology (RSM) was used to optimize key factors, *i.e.*, pH, temperature, enzyme concentration, and reaction time. Small rotatable central composite design (SRCD) with eight runs for axial and factorial points and five replicate runs at center points was augmented to estimate key factors, their respective interactions, and pure error. Each factor was investigated at five levels ($-\alpha$, -1, 0, +1, $+\alpha$ (α=1.682)) in a randomized order, as expressed in Table 1. All data were manipulated in Design Expert (Version,

8.0.7.1) statistical software for design selection, robustness, and graphical and numerical optimization. The effects of enzymatic treatment parameters and their interactions were calculated at a 95% confidence interval to produce mean values. Four responses including extract yield (yield), total phenolic contents (TPC), Trolox equivalent antioxidant capacity (TEAC), and DPPH radical scavenging capacity were modeled by the following second-order polynomial equation,

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X^2 + \sum_{i>1}^{k} \sum_{j}^{k} b_{ij} X_i X_j \quad (1)$$

where Y denotes the response to be optimized, b_0 is the intercept, $\sum_{i=1}^{k} b_i X_i$ is the linear effect of variables, $\sum_{i=1}^{k} b_{ii} X^2$ is the quadratic effect, and $\sum_{i>1}^{k} \sum_{j}^{k} b_{ij} X_i X_j$ is the interaction between parameters.

Enzymatic treatment

Accurately weighed 5 g of *C. limetta* peel (CLP) was placed in a 250 mL Erlenmeyer flask, diluted with 10 mL of phosphate buffer, blended with a 1:2:1 ratio of pectinex, celluclast, and alcalase enzymes, and incubated in an orbital shaker (120 rpm) at the required temperature for a specific time interval, as given in Table 1. Then, the enzyme cocktail was deactivated by heating at 90 °C for 5 min and the contents were degassed in an ultrasonic reactor (UTECH, Albany, New York, USA).

Scanning electron microscopy (SEM)

The ultrastructure of enzymatically hydrolyzed CLP samples was determined by field emission scanning electron microscopy (FESEM). The samples were dried using liquid nitrogen, fixed on a specimen holder using magnetic tape, and coated with palladium in a sputter coater. The morphological changes were examined using a MIRA 3 (Tescan, Czech Republic) SEM operating at an accelerating voltage of 3 kV.

Extraction total phenolic contents (TPC)

The digested CLP samples were extracted with 100 mL of methanol in an orbital shaker (Pamico, Pakistan) to collect liberated phenolics. The final extracts were further concentrated under reduced pressure using a rotary evaporator (EYELA, N-N series, Tokyo, Japan). The TPC in obtained CLP extracts was assessed using Folin-Ciocalteu reagent as described by Sulaiman *et al.* (2011) with slight modifications.

HPLC profile of phenolic compounds

Reverse-phase high-performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD) was used to characterize phenolic compounds in CLP extracts, as previously documented by Abadio Finco *et al.* (2012). Briefly, 50 mg of extract was refluxed at 95 °C in 50 mL of 1% acidified methanol containing 0.5 mg/mL BHT as a preservative antioxidant, cooled to room temperature, centrifuged at 5000 rpm for 10 min, and allowed to settle in a separating funnel. Finally, 1 mL of the upper layer was collected, degassed, filtered through a 0.45-µm filter (Millipore), and injected into the RP-HPLC-DAD. Phenolic compounds in CLP extracts were eluted and quantified using a Shim-Pack CLC-ODS C-18 column (250 mm x 4.6 mm x 5 µ i. d.) in a Shimadzu LC-10A HPLC system (Japan) coupled with a DAD at 280 nm. The data were processed using a CSW32 (data-apex) chromatography station. Gradient mode (0 to 15 min 15% B, 15 to 30 min 45%

B, and 30 to 45 min 100% B) mobile phase consisting of A (H_2O:CH_3COOH 94:6) and B (acetonitrile 100%) at a flow rate of 1.0 mL/min was used to elute the phenolic constituents.

Trolox equivalent antioxidant capacity assay (TEAC)

The extracts of CLP obtained through the observed conditions were evaluated for their antioxidant contents by measuring their Trolox equivalent antioxidant capacity (TEAC) (Arts *et al.* 2004). $ABTS^{\bullet+}$ was generated by mixing 7 mM ABTS and 2.45 mM potassium persulphate (0.5/1; v/v). The mixture was kept in the dark for 8 h and diluted with 80% ethanol until it produced an absorbance of 0.700 ± 0.050 at 734 nm. To 190 µL of diluted ABTS radical cation solution, 10 µL of extract (50 mg/mL) was added and allowed to stand for 6 min. Trolox (known antioxidant) was used as a positive control, and antioxidant potential was expressed as mg TE/g of *C. limetta* peel extract.

DPPH• scavenging assay

The free radical scavenging activity of CLP extracts was appraised following a previously documented procedure (Chen *et al.* 2013). Briefly, 100 µL of freshly prepared DPPH (1 mg/mL) was mixed with 110 µL each of 1, 0.1, 0.01, and 0.001 mg/mL extract. After a 15 min incubation at room temperature, absorbance was measured at 517 nm using a spectrophotometer,

$$\text{Inhibition } \% = \frac{\Delta_{Control} - \Delta_{sample}}{\Delta_{Control}} \times 100 \quad (2)$$

where $\Delta_{Control}$ indicates the absorbance of solution containing only the $DPPH^{\circ}$, whereas Δ_{sample} is the absorbance of the sample reaction. The effective dose of 50% inhibition (IC_{50}) was also obtained from a plot of percentage inhibition *versus* extract concentration. All experiments were performed in triplicate, and mean values were thus calculated against ascorbic acid and butylated hydroxyl toluene (BHT) as positive controls.

RESULTS AND DISCUSSION

Experimental Design Evaluation

Response surface methodology (RSM), a common statistical approach, was used to optimize the key factors involved in the enzymatic treatment of CLP. In this context, enzyme concentration (EC), incubation temperature (*T*) and time (*t*), and pH were investigated at five different levels, as described in Table 1, to augment the extraction yield, phenolic content, and antioxidant activities. Based on the differences between observed responses, an experimental error with a 95% confidence interval for the means was determined to evaluate the effect of each variable and their interaction. The model statistics and analysis of variance (ANOVA) data (Table 2) revealed that the applied model was significant ($p < 0.05$) with a good fit and that multivariate responses can be generated by a second-order polynomial equation. The values of adjusted R^2 were greater than 50%, which verifies good agreement between actual and observed responses. Coefficients of variation ranging from 1.63 to 3.35 further authenticated that the results obtained are quite reliable (Ravikumar *et al.* 2006).

Response surface methodology has been frequently used to optimize single-response studies (Aissa *et al.* 2007; Amouzgar *et al.* 2010; Aouadhi *et al.* 2013). Nevertheless, this study encounters the situation where the factors investigated control almost all the responses to be observed. This difficulty can be resolved by the use of visual display and the desirability approach (Wei *et al.* 2009). In this methodology, the measured responses are further transformed into a dimensionless scale of desirability with values from 0 to 1. A desirability value of zero indicates a totally adverse set of experimental conditions, whereas a value of unity denotes the most suitable experimental conditions.

Table 1. Observed Response of Assays Conducted to Evaluate Enzymatic Treatment Parameters (Actual and Coded) at Different Levels (Actual and Coded) in Small Central Composite Design (SCCD)

		Factors (real and coded) Investigated				Response observed			
Treatment	Point type	*E* (%) (A)	*T* (°C) (B)	*t* (Min) (C)	pH (D)	Extract yield[L]	TPC[M]	TEAC[N]	DPPH[O]
2	Center	3.5 (0)	45(0)	75(0)	6.5(0)	76.84	102.00	177.00	96.00
5	Center	3.5(0)	45(0)	75(0)	6.5(0)	75.57	98.00	176.00	97.00
6	Center	3.5(0)	45(0)	75(0)	6.5(0)	76.33	97.00	176.29	99.00
14	Center	3.5(0)	45(0)	75(0)	6.5(0)	76.25	99.64	176.29	96.00
17	Center	3.5(0)	45(0)	75(0)	6.5(0)	76.11	100.00	172.00	93.00
3	Axial	3.5(0)	45(0)	75(0)	5.0(-α)	46.81	73.82	146.68	72.12
7	Axial	5.0 (+α)	45(0)	75(0)	6.5(0)	72.00	61.60	135.68	67.59
9	Axial	3.5(0)	45(0)	30(-α)	6.5(0)	74.00	83.74	157.43	89.73
10	Axial	3.5(0)	45(0)	120(+α)	6.5(0)	63.57	90.00	164.93	88.23
11	Axial	3.5(0)	60(+α)	75(0)	6.5(0)	70.07	59.62	134.68	63.00
15	Axial	3.5(0)	45(0)	75(0)	8.0(+α)	54.31	82.32	155.68	86.36
19	Axial	3.5(0)	30(-α)	75(0)	6.5(0)	64.79	53.50	130.68	61.24
21	Axial	2.0(-α)	45(0)	75(0)	6.5(0)	58.50	76.89	150.68	69.35
1	Factorial	4.0(+1)	55(+1)	100(+1)	5.5(-1)	41.00	48.21	130.21	47.21
4	Factorial	2.5(-1)	40(-1)	100(+1)	5.5(-1)	61.71	64.53	137.82	66.53
8	Factorial	2.5(-1)	55(0)	50(-1)	7.5(+1)	57.67	48.74	129.71	58.38
12	Factorial	2.5(-1)	40(-1)	50(-1)	5.5(-1)	51.00	44.06	119.71	52.00
13	Factorial	4.0(+1)	40(-1)	50(-1)	7.5(+1)	62.23	88.93	146.71	85.00
16	Factorial	4.0(+1)	40(-1)	100(+1)	7.5(+)	59.72	79.56	143.82	82.36
18	Factorial	4.0(+1)	55(+1)	50(-1)	5.5(-1)	65.04	55.91	134.71	62.32
20	Factorial	2.5(-1)	55(+1)	100(+1)	7.5(+1)	47.03	65.04	136.82	73.69

[L, M, N] and [O] are extract yield (g/100 g DW), total phenolic contents (mg GAE/g DW), antioxidant activity (mmol TE/g DW), and DPPH radical scavenging potential (%), respectively

Extraction Yield

The regression model used to predict extraction yield, TPC, and antioxidant activities was significantly controlled by the linear effect of parameters, their interactions,

and quadratic terms (Table 2). It can be concluded from Fig. 1 that all the factors investigated significantly affected the recovery of phenolic antioxidants in CLP.

Table 2. Analysis of Variance of Estimates (ANOVA) for Extraction Yield, TPC, TEAC, and DPPH Radical Scavenging of CLP

	Yield (g/100g DW CLP)		**TPC mg GAE/g DW CLP**		**TEAC mg TE/g DW CLP**		**DPPH (IC_{50} mg/mL)**	
Source	F-ratio	*p*-value[a]	F-ratio	*p*-value[a]	F-ratio	*p*-value[a]	F-ratio	*p*-value[a]
Model	177.54	< 0.001	194.51	< 0.001	176.63	< 0.0001	69.33	< 0.0001
A	101.22	< 0.001	43.32	0.0006	41.50	0.0007	0.29	0.6069
B	15.49	0.007	6.94	0.0389	2.95	0.1366	0.29	0.6069
C	121.52	< 0.001	24.80	0.0025	25.03	0.0024	1.28	0.3018
D	31.26	0.001	13.37	0.0106	14.94	0.0083	19.28	0.0046
AB	16.93	0.0063	46.73	0.0005	3.33	0.1179	13.85	0.0098
AC	52.34	0.004	134.27	< 0.001	49.02	0.0004	53.84	0.0003
AD	49.29	0.004	104.31	< 0.001	13.03	0.0112	23.14	0.0030
BC	176.67	< 0.001	0.29	0.6101	7.33	0.0353	3.25	0.1216
BD	11.78	0.0139	143.96	< 0.001	85.92	< 0.0001	9.15	0.0233
CD	6.88	0.0394	1.58	0.2552	4.07	0.0902	4.17	0.0871
A^2	306.52	< 0.001	620.65	< 0.001	729.91	< 0.0001	271.47	< 0.0001
B^2	206.34	< 0.001	1257.78	< 0.001	1276.86	< 0.0001	410.52	< 0.0001
C^2	135.12	< 0.001	105.01	< 0.001	145.80	< 0.0001	18.07	0.0054
D^2	1496.11	< 0.001	308.63	< 0.001	415.17	< 0.0001	101.14	< 0.0001
Lack of Fit	0.28	0.5458	0.18	0.8438	0.032	0.9691	1.36	0.3552
R^2		0.9974		0.9978		0.9976		0.9939
Adj R^2		0.9914		0.9927		0.9919		0.9795
Pred R^2		0.7003		0.9860		0.9920		0.8562
CV		1.63		2.19		3.35		3.00

[a]The *p* value was used as a tool to check the significance of each coefficient. Very small *p* values for the linear and quadratic terms indicate the significant contribution of these predictors in the fitted model

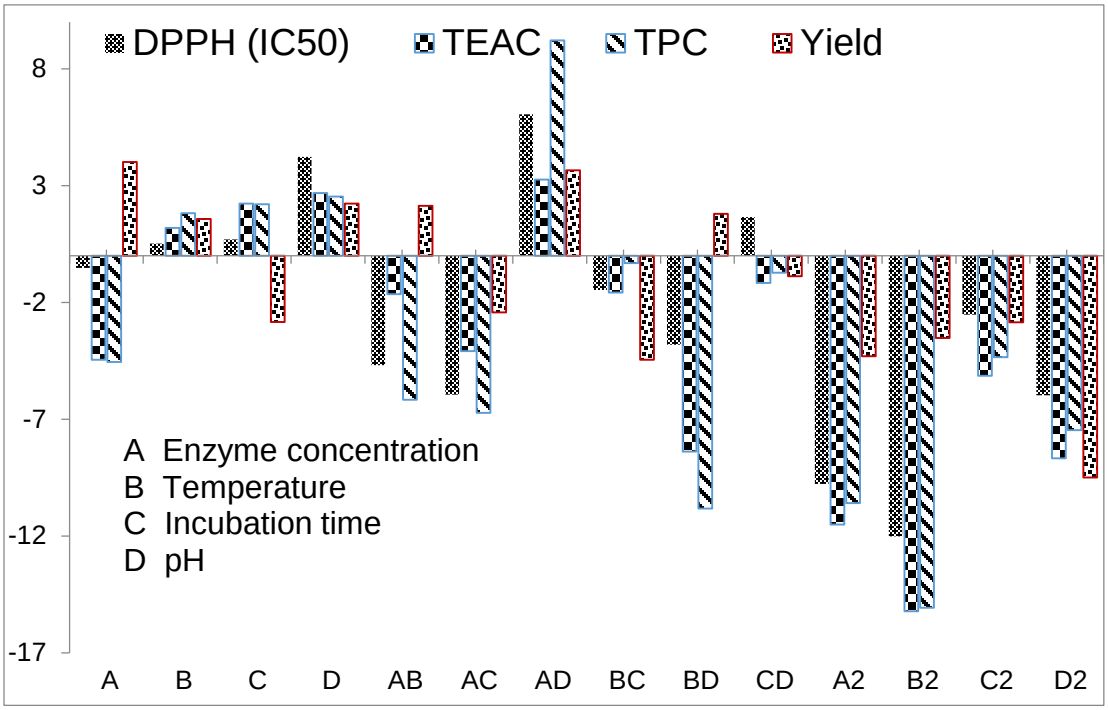

Fig. 1. Comparison of the effects of enzymatic treatment parameters towards extraction yield and antioxidant activities of CLP

The synergism between experimental parameters and stationary points can be better understood by constructing a three-dimensional visualization of the model (Fig. 2). It can be seen that all studied variables except incubation time (Fig. 2e) lay within the selected experimental region. Figures 2a, 2c, and 2f indicate a sharp change in extraction yield in response to enzyme concentration, pH, and temperature. An incubation time of more than 75 min (Fig. 2f) did not affect the extract yield.

Table 1 shows that the extract produced ranged from 41.00 to 76.84 g/100 g DW, whereas that of the control (without enzyme pre-treatment) was 31.40 ± 1.26 g/100 g DW of CLP. Hence, incorporation of enzymes under suitable conditions can enhance the liberation of phenolic antioxidant by more than two-fold. The reason for higher extraction might be the liberation of bound phenolic compounds, which may include phenolics entrapped in cellulosic microfibrils via either hydrophobic (by means of covalent bonding with polysaccharides) or hydrogen linkages with cellulosic microfibrils (Acosta-Estrada *et al.* 2014). Bound phenolic compounds constitute about 24% of the total phenolic constituents, and conventional aqueous or organic solvents (such as 80% ethanol) may not be able to liberate bound phenolics as endeavored in previous reports (Ramful *et al.* 2010; Rodríguez-Rivera *et al.* 2014).

The validation experiments conducted under the most suitable conditions with a maximum desirability of 0.9726 at enzyme concentration of 3.31%, temperature of 45.24 °C, pH of 6.60, and incubation time of 85.57 min resulted in an extract yield of 76.51 ± 1.27 g/100 g (Table 3).

Table 3. Experimental Validation of Most Suitable Enzymatic Treatment Conditions Indentified by Selected Statistical Model

Sr. No.	Maceration parameters optimized				Responses observed			
	E C (%) (A)	*T* (°C) (B)	*t* (Min) (C)	pH (D)	Yield[L]	TPC[M]	TEAC[N]	DPPH[O]
1	3.30	45.00	84.50	6.60	75.51	101.48	176.41	96.56
2	3.30	45.00	84.50	6.60	76.08	99.56	178.22	95.32
3	3.30	45.00	84.50	6.60	79.93	99.34	175.98	95.64
average					76.51± 1.27	100.13± 1.18	176.90± 1.33	95.84 ±0.64
Results predicted with maximum desirability					76.22	100.48	176.51	96.56

[L, M, N] and [O] are extract yield (g/100g DW), total phenolic contents (mg GAE/g DW), antioxidant activity (mg TE/g DW) and DPPH radical scavenging potential (%), respectively.

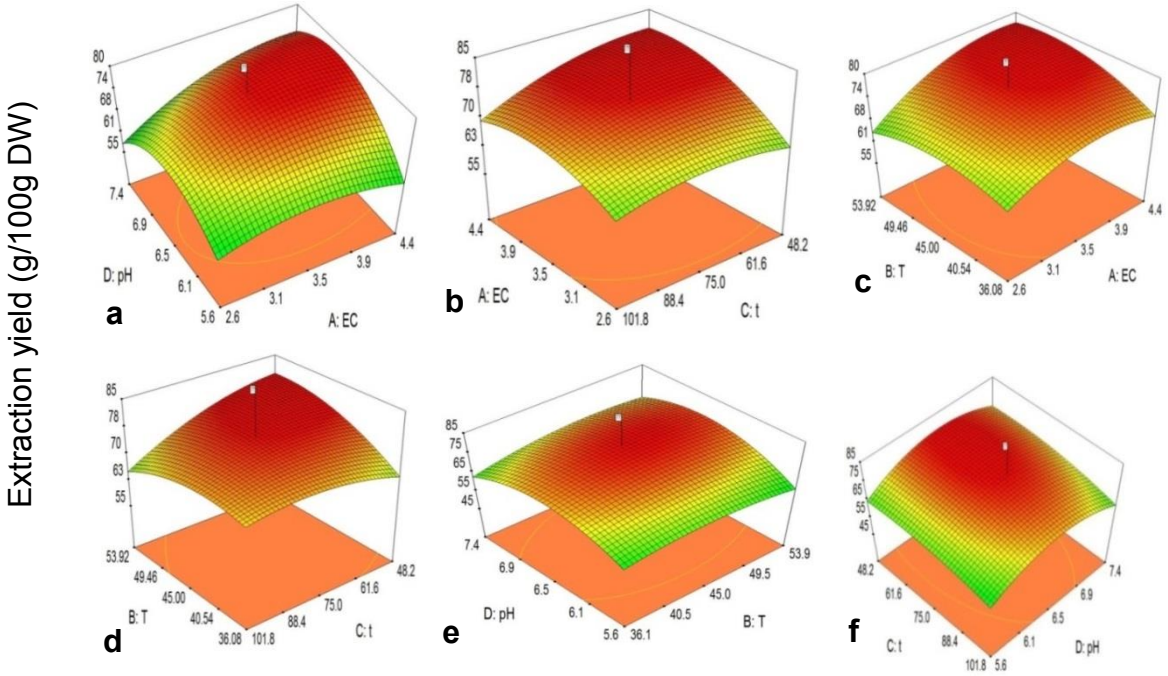

Fig. 2. Three dimensional response plot of observed yield (%) against different optimized enzymatic treatment parameters and their interaction

Scanning Electron Microscopy (SEM)

The increased extraction yield as compared to the control must be attributed to the digestive power of the enzyme cocktail utilized, which hydrolyzed CLP (Fig. 3) and improved the soaking and distribution power of the methanol extraction media. It can be easily seen that the cells of untreated CLP were intact (Fig. 3a), whereas those treated were efficiently broken down by the enzymatic treatment (Fig. 3a). The destruction of the cell wall improved the distribution power of solvent and significantly enhanced the recovery of entrapped phenolic compounds.

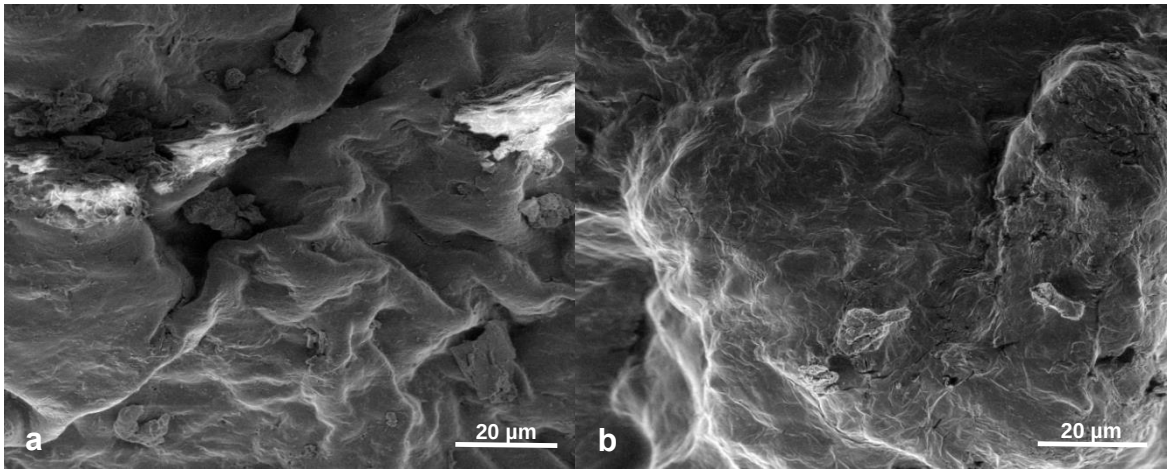

Fig. 3. Morphological micrograph of (a) CLP and (b) enzymatically hydrolyzed CLP

Phenolic Compounds in CLP

Awareness regarding the cumulative biological characteristics of phenolics, including anti-inflammatory, antibacterial, vasodilatory actions, anticarcinogenic, antiviral, antithrombotic, antiallergic, and hepatoprotective effects (Del Bano *et al.* 2006; Jayaram and Dharmesh 2011; Turner *et al.* 2005), have led to the extensive study of the bioactivities of these plant materials (Haggag *et al.* 2011). During the present study, plant cell walls were enzymatically treated by cellulasic, pectinase, and hydrolytic enzymes (acid cellulase), which ultimately improved the liberation of glycosidically entrapped phenolic compounds. The amount of TPC in CLP extracts estimated using FCR was found to range from 44.06 to 102.00 mg gallic acid equivalents (mg GAE/g DW). The statistical evaluation indicated that all the factors investigated affected the recovery of antioxidant phenolics, and the contribution of enzyme concentration and pH was significantly higher than other enzymatic treatment parameters tested (Fig. 1).

Optimization data regarding total phenolic contents revealed that incubation can be adopted flexibly within the range of 30 to 100 min. The validation results were obtained under the most suitable experimental conditions, *i.e.*, enzyme concentration 3.31%, pH 6.60, temperature 45.24 °C, and incubation time 85 min, and were in synchronization with results predicted by the applied model. Because enzymatic treatment and extraction conditions may affect the quality of liberated phenolics, in addition to FCR determination, a more reliable approach, *i.e.*, RP-HPLC-DAD, was used to characterize phenolic compounds. The major phenolic compounds found in CLP extracts obtained under optimum conditions of enzymatic treatment are given in Table 4. The presence of potential phenolic compounds in CLP extracts certified that pre-enzymatic treatment under optimum conditions enhanced their liberation and recovery. The increased liberation rates of phenolic compounds may be attributed to the compositional profile of the enzyme cocktail, especially because of the presence of pectinase, protease and α-amylase, β-glycosidase, and hydrolytic units in acid cellulase. The HPLC-DAD characterization and quantification of crude CLP extracts obtained under optimum enzymatic treatment indicates the presence of potential phenolic compounds, especially phenolic acids (Table 4). Chlorogenic (1427.04 µg/g), *p*-coumeric (277.36 µg/g), ferulic (217 µg/g), and syringic acids (205.36 µg/g) were found to be major phenolic acid constituents of the extracts. Most of these phenolic acids have been approved as food additives (folic acid) that not only prevent lipid peroxidation of food but also confer therapeutic benefits against various oxidative stress-

related diseases such as diabetes and cancer and cardiovascular and neurodegenerative disorders (Srinivasan *et al.* 2007).

Table 4. Major Phenolics Identified in Enzyme Assisted Solvent Extracts of CLP

Sr. No	Retention Time (min)	Phenolics	Concentration (µg/g of extract)
1	2.48	Protocatechuic acid	19.71
2	3.09	Quercitin	73.57
3	4.94	Gallic acid	ND
4	12.94	Caffeic acid	ND
5	13.07	Vanillic acid	10.37
6	14.95	Myricetine	112.15
5	15.22	Chlorogenic acid	1427.04
7	16.25	Syringic acid	205.36
8	17.97	*p*-coumaric acid	277.36
9	20.51	*m*-coumaric acid	115.29
10	22.92	ferulic acid	217.84
11	27.22	Sinapic acid	10.02

ND: not detected

Antioxidant Activities (AA) of CLP Extract

The methanol extracts of enzymatic pre-treated CLP were evaluated for their antioxidant character using model ABTS and DPPH *in vitro* assays. The ABTS radical cation oxidants produced by the reaction of ABTS with potassium persulphate and scavenged by the extracts were quantified spectrophotometerically at 734 nm (Sashidhara *et al.* 2011) *versus* Trolox (Vitamin E) as a positive control. Values obtained are expressed as mg of Trolox equivalent ((mg TE)/g of dry extract (DW)).

The observed values of Trolox equivalent antioxidant capacity (TEAC) ranged from 119.71 to 177.00 mg TE/g DW, as indicated in Table 1. The maximum antioxidant activity at 177.00 mg TE/g DW was exhibited by the CLP treated with 3.5% enzyme cocktail at 45 °C, 6.5 pH, and 75 min treatment time. Overall, observed values indicated that antioxidant activities were significantly affected by the treatment conditions applied. The most suitable conditions to obtain extracts with optimal TE antioxidant potential (176.90 ± 1.33 TE/g DW) as recommended by the selected statistical approach were 3.31% EC, 45.24 °C, 6.60 pH, and 84.50 min incubation time.

The antioxidant activity in terms of radical scavenging potential was evaluated by using a stable organic free radical, *i.e.*, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The tabulated data regarding DPPH radical scavenging capacity (%) under optimum enzymatic treatment conditions indicates that DPPH radicals were potentially scavenged by extracts of CLP (95.84 ± 0.64). All values observed under the conditions with maximum

desirability were in agreement with the validation experiment results (Table 3). A comparison with previously available data regarding conventional solvent extraction and incorporation of acidic or alkaline hydrolysis to extract phenolic compounds from citrus species (Bushra Sultana *et al.* 2008; Kanaze *et al.* 2009; Yaqoob *et al.* 2014) indicate that extracts obtained from enzymatically treated CLP scavenged the DPPH radical more efficiently. Hence, the utilization of enzymes for enhanced recovery of phenolic compounds produced extracts with higher levels of retained antioxidant character as compared to conventional solvent extracts (CSE). The incorporation of pre-enzymatic treatment produced the optimal predicted response of DPPH radical scavenging, which was observed at 2.33% EC, 6.72 pH, 50.06 °C, and 30.0 min incubation time.

CONCLUSIONS

1. Enzymatic treatment parameters were optimized for enhanced liberation of antioxidant phenolics from underutilized *C. limetta* peel (CLP).
2. The results indicated that pre-treatment with cocktail enzyme doubled the liberation of crude phenolics.
3. The extracts obtained exhibited ample level of antioxidant activities.
4. Furthermore, RP-HPLC-DAD characterization of extracts indicated the presence of potent phenolic acids especially ferulic acid and chlorogenic acid.
5. Enzyme assisted solvent extracts might be good candidates for valorization of underutilized *C. limetta* peel into phenolics for value addition.

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