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MICROWAVE METHOD-A NOVEL GREEN TECHNIQUE FOR EXTRACTION OF BIOACTIVES FROM THE WASTE OF PINEAPPLE BY OPTIMIZING THE CONDITIONS

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Abstract: In the recent years the usage of natural bioactive sources are increasing as they add more value to the food industries as well in the pharmaceuticals to manufacture drugs. Waste accumulation is a common problem as they cause serious problems to the environment. In the present study the extraction of bioactive compounds from the pineapple peel and core was done using microwave technique using ethanol as solvent and the extraction conditions were optimized using response surface methodology (RSM) by using the variables time (min), power (watt) and ethanol concentration(%) these parameters were varied to get the best condition for the extraction of protein, phenolic content and antioxidants. Further for the optimized condition of protein purification was carried out using acetone precipitation and the bromelain activity was obtained. SDS-Page determined the protein pattern to be 23 kDa and the bromelain presence was sorted using casein activity staining method which confirmed the presence of bromelain in the peel and core. Therefore microwave assisted extraction can be one of the quickest novel green technique for the extraction of bioactives and enzymes.
Keywords: Pineapple waste, Microwave assisted extraction, Optimized condition, Purifications, Bioactives, SDS-Page.

INTRODUCTION

Microwave extraction is one of the trending novel systems and it can accomplish higher extraction yield with low dissolvable utilization and extraction time. The use of microwave power for the extraction of bioactive mixes brought about fast and uniform warming of plant material and furthermore the dissolvable which is used (Eshamah, Han, Naas, Rieck, & Dawson, 2013). Its mechanism is based on the principle of dipole rotation and ionic conduction. The peel and core of the pineapple is considered to be a waste which is obtained from the processing plants such as the pineapple juice, canned products and as ready to serve fruit. The waste accounts nearly 50% which includes the crown, stem, peel and core. Apparently, the waste has a lot of valuable phenolic compounds and the proteolytic enzyme bromelain which has potential uses in pharmaceutical and food industries (Bitange, Wang, Xu, & Zhang, 2008). Furthermore, the disposal of waste is a growing issue in the environment be-

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cause of the microbial spoilage which is an impact of illness to the surroundings. A clean green technique is necessary to isolate the bioactives, prevent the environment using an integrated approach in the scene of recycling, and reuse in the waste recovery.

The objective of the present study is to extract the bioactives using ethanol as solvent, total phenolic content (TPC), total protein and Ferric reducing antioxidant power (FRAP) using three independent variable time(min), power (watt) and ethanol concentration (%) by using the response surface methodology (RSM) to characterize the bio actives and obtain the maximum yield. Then after the purification of protein was carried out using Acetone precipitation and the protein pattern was determined using SDS-Page analysis.

MATERIALS AND METHODS

Materials:

Raw materials:

Raw materials used in this experiment is the pineapple peel and core obtained from the "Prime Products Industry Co.Ltd" Chonburi Province, the company generates a waste of 13 % percent of the pineapple waste.

Chemicals:

All chemicals used were of analytical grade. Bradford dye reagent (PRD.0.ZQ5.10000050486) ,Sodium acetate, Glacial acetic acid(ARK2183), EDTA, Ethanol, HCl, Folin-ciocalteau reagent (Code No: -03870), Ferric Chloride, Gallic acid, TPTZ (2, 4, 6-tripyridyl-s-triazine), Potassium phosphate buffer, Glycerol, Ferric chloride, Methanol (CAS No: 67-56-1), Sodium carbonate (CAT No:463), Acetone, L-Tyrosine (CAS No:60-18-4), Casein (CAS No: 9000-71-9), Sodium carbonate solution, Sodium acetate buffer, Calcium acetate buffer, L-cysteine (PUB chem 24901592), Trichloroacetic acid (PubChem CID 6421) Sodium dodecyl sulfate (PubChem CID 3423265), Hydrochloric acid (PubChem CID 313) Sodium hydroxide (PubChem CID 14798).

Methodology:

Sample preparation:

The peel and core were cleaned using distilled water and dried in a hot air oven at 40 □ for 48 hrs and then it was grinded and kept at 4□ for further extraction.

Microwave Assisted Extraction:

The lab scale microwave (Samsung, 1.6 cu.ft, 1000W) contained the heating unit, a flask for extraction (1L), condenser and a vacuum machine. A 5g of sample with a fixed ratio of 1:10 g/mL was kept in the flask and inserted into the microwave chamber and power was supplied

at (100, 180 and 300 W) for the time (5, 10 and 15 minutes) and different ethanol concentration (0, 20 and 40%). The conditions were optimized using Box Benkhen by the Design expert (trial version 10.0.1, stat-Ease Inc, Minneapolx, MN, USA) and gave 15 runs of trial. Both the peel and the core was extracted under these conditions. Then the extract was further filtered, centrifuged and analyzed for the total protein, TPC and the FRAP.

Table 1: Independent factors with their coded and actual values used for optimization in the microwave

Independent factors	Symbol	Coded Levels		
		-1	0	1
Time (min)	A	10	20	30
Amplitude (%)	B	60	80	100
Ethanol concentration (%)	C	0	20	40

Purification by Acetone precipitation:

To purify the protein sample, acetone was taken and cooled to (-20 º C) . Protein sample was added to the cold acetone in the ratio (1:4) and vortexed thoroughly. The sample was maintained at - 20 º C for 60 minutes and then centrifugation at 13,000 g for a time interval 10 min (Ota et al., 1964). The supernatants after centrifugation were thrown and tubes which has pellet was in inverted direction for 15 min in order to remove acetone from the pellet which can be of excess. Re Dissolving of protein pellet was done in 0.01 mol/L phosphate buffer (pH 7.0). The solution was dialyzed against 0.01 mol/L phosphate buffer (pH 7.0) using a 1 kDa dialysis membrane for 14 h at 4 ºC (Jain & Anal, 2016).

Quantitative analysis:

Total Protein content: BSA reagent was made by diluting the reagent with distilled water in the ratio of 1:4.100 µl. and 5 ml of Bradford dye was added to the sample solution. Then it was vortexed thoroughly and mixture was kept for incubation at ambient room temperature for 15 min and then absorbance was measured at 595nm(UV-UNICAM, ALVA, U.K) (Bradford, 1976).

Total phenolic content: Folin-Ciocalteau method (Kukrić et al., 2012)was used to determine the amount total phenolic present in the sample. 0.5ml of the diluted sample was taken and added with folins reagent of 2 ml which was freshly prepared at 1:10 ratio, to the mixture 7.5% sodium carbonate of 4 ml was added and kept for incubation for 30 minutes long and

using the UV spectrophotometer the absorbance was measured at 765 nm (UV-UNICAM, ALVA, U.K).

Ferric reducing antioxidant power: Ferrous sulphate was used as the standard to determine the antioxidant power. To 100 µl of the sample, the FRAP reagent of 3 ml was mixed, vortexed and kept for incubation during a time period of 3 min and the absorbance was measured at 593 nm in the UV spectrophotometer (UV-UNICAM, ALVA, U.K).

Bromelain activity: The determination of bromelain activity was by done by casein digestion method by the presence of cysteine and EDTA (Murachi, 1976) using casein as a substrate.

The enzyme activity was calculated using the equation

Activity (CDU/ml) $= \boldsymbol{Et - \frac{Eb}{Es} * concentration\ of\ standard\ Ltyrosine * \frac{Vr}{Tr} * Df}$

E_t -absorbance of enzyme sample, E_b - absorbance of enzyme blank and E_s - absorbance standard L- tyrosine, D_f .dilution factor, Vr - reaction volume, tr - reaction time.

Electrophoresis Analysis: (Bio-Rad Laboratories, Inc., Richmond, CA, USA)

The molecular weight and protein pattern determination was determined using the SDS page. The protein samples obtained from the optimized condition was subjected to the gel electrophoresis (Ketnawa, 2011). Further activity staining was performed to confirm the bromelain activity (García-Carreño, Dimes, & Haard, 1993).

RESULTS AND DISCUSSION

Fitting the model for the extraction of bioactive components from pineapple peel

In the Microwave assisted extraction (MAE), microwaves are generated through two mechanisms i.) ionic polarisation ii.)dipole interaction. The microwave power is being transmitted through a material which is absorbed and converted into heat.The direct heating helps in reducing the extraction time and also the extraction yied is increased. From the extraction conditions the protein content was in range from 2804.79 – 4903.04(µg/g), the phenolic content was found to be 3856.31 – 9894.66 (µg GAE/g) and the FRAP value was found to be 704.07- 1207.66 (µmol Fe (II)/g of extract). The FRAP value obtained from the microwave conditions was higher due to the microwaves which has the ability to disrupt hydrogen bond networks. The microwave-induced dipole rotation of molecules, and the migration of ions that enhance the penetration of solvent in to matrix, disrupts the cell wall and releases the intracellular product, allowing for the extraction of different components (Phongthai, Lim, & Rawdkuen, 2016). The total phenolic content in the potato peel using methanol as solvent in the MAE was found to be 3.94 ± 0.21 mg /g dw (Singh et al., 2011)

which is correlated with our study. The ANOVA was done to determine the significance and the model used was quadratic and the R^2 value for the protein, TPC and FRAP was found to be (0.8639, 0.5966, 0.4254) respectively. The obtained ANOVA revealed that there was no significance difference between each variables such as the time, power and ethanol concentration on the extraction of protein, phenolic and antioxidants. In case of phenolic content,the lack of fit was found to be significant ($p<0.05$)The optimum extraction conditions was found to be for time 13 min , Power 252 Watt and ethanol concentration 40% yielded 4903.14 (µg/g) 8273.17 (µg GAE/g) and 1090.28 µmol Fe(II)/g with a desirability 0.825. The regression equation represents an empirical relation between the responses and the independent variables, here Y_1, Y_2 and Y_3 represent the protein content, TPC and FRAP content of peel and X_1, X_2 and X_3 are the actual values of the independent variables.

$Y_1=3200.73+431.107X_1+347.924X_2+400.19X_3+269.806X_1X_2+109.35X_1X_3+274.15X_2X_3+297.327X_1^2+277.857X_2^2+308.035\ X_3^2$ ……. (1)

$Y_2=7547.68+313.684\ X_1+948.258\ X_2+965.243\ X_3+220.467\ X_1X_2+292.217X_1X_3-1395.17\ X_2X_3-165.744\ X_1^2-455.625X_2^2-290.484\ X_3^2$…… (2)

$Y_3= 955.586-53.9704\ X_1+63.1037\ X_2+80.2966\ X_3+22.8331\ X_1X_2+46.705X_1X_3+18.3157\ X_2X_3-39.2012\ X_1^2-32.1375\ X_2^2+35.1162\ X_3^2$…….. (3)

Further with the predicted value, experiment was carried out to validate the results which confirmed the validity those were further used for purification process.Response surface graphs were generated by keeping one variable as constant and other two were varied to show interactive effects.

a. b. c.

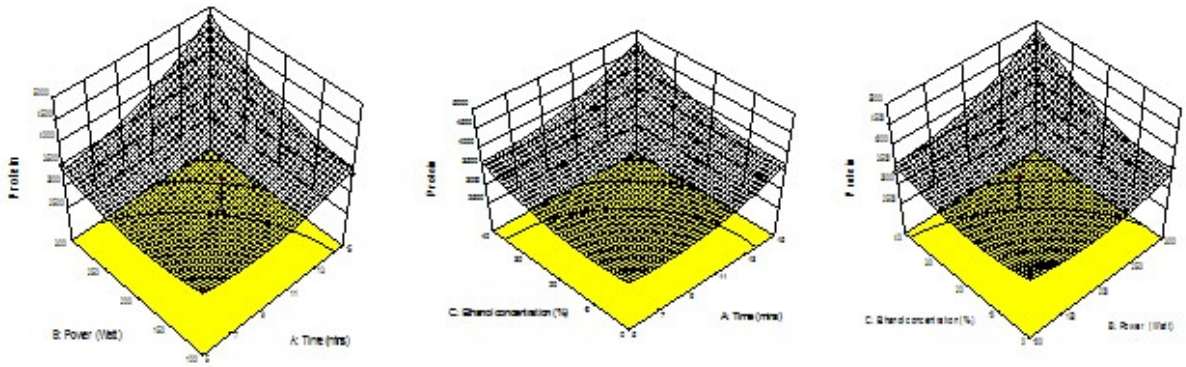

Fig 1: Response surface graphs showing effect of time(min), Power (watt) and ethanol concentration (%) on protein content (µg/g of crude extract) from pineapple peel a.) Time & power b.) Ethanol concentration & time c.) Ethanol concentration & power

a. b. c.

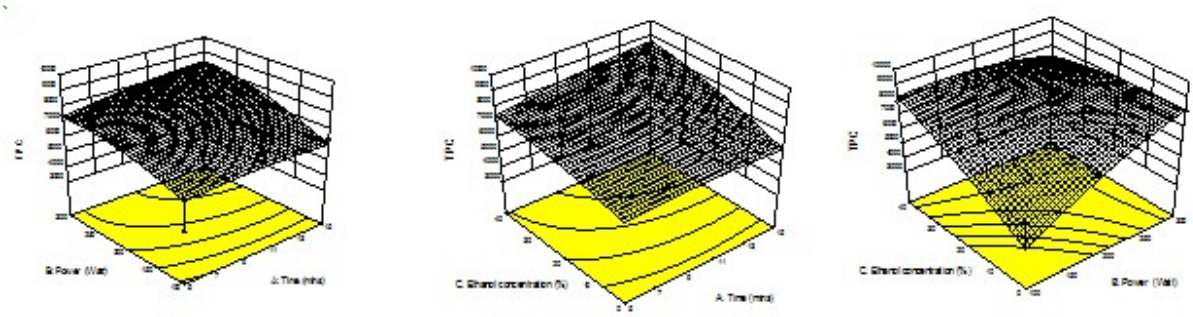

Fig 2: Response surface graphs showing effect of time(min), Power (watt) and ethanol concentration(%) on phenolic content(µg of GAE/g)from pineapple peel a.)Time & power b.) Ethanol concentration & time c.)Ethanol concentration & power.

a. b. c.

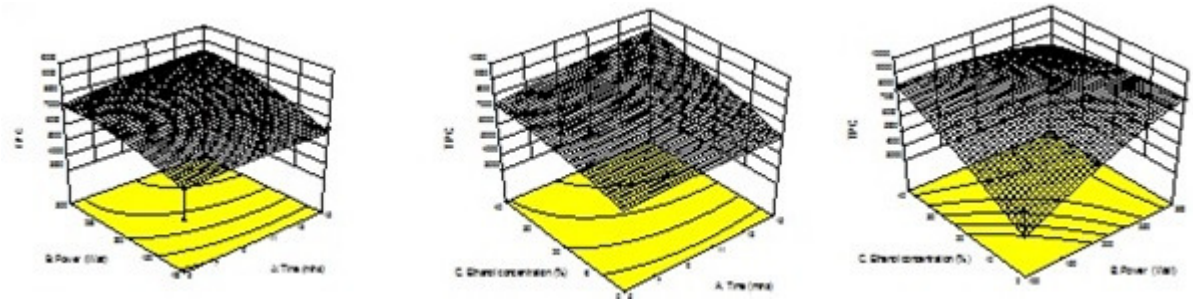

Fig 3: Response surface graphs showing effect of time(min), Power(Watt) and ethanol concentration(%) on antioxidant content from pineapple peel a.) Time & power b.) Ethanol concentration & time c.) Ethanol concentration & power

Fitting the model for the Extraction of bioactive components from pineapple core :

The extraction of bioactive components such as Protein, TPC and antioxidant content from the core of the pineapple using MAE obtained. The model used to determine the extraction of bioactive compounds were quadratic and has an R^2 (0.7471, 0.7856, 0.5183)for protein, TPC and FRAP respectively. From the ANOVA it was predicted that the varibles time, ethanol concentration and power did not have any significant difference on the Protein and FRAP value and so is the model term. While the phenolic content possesed some significant difference and also the lack of fit was found to be significant. From the extraction process conditions the protein content ranged from (2749.74 – 3860.03 µg/g) , the phenolic content was found to be (9169-216572 µg GAE/g) and the FRAP value (141.39 – 285.37 µmol Fe(II)/g). The tpc was observed to be higher this due to the MAE utilizes microwave vitality and solvents to concentrate target mixes from different grids. The exceedingly limited

temperature and weight can bring about particular movement of target mixes from the material to the surroundings at more quick rate and with comparative or better recuperations contrasted and customary extraction, with the primary focal points of lessening both extraction time and dissolvable utilization. The optimized condition obtained with the maximised yield of protein 3886.92µg/g TPC161281.21µg GAE/g and FRAP 239.18µmol Fe(II)/gwith the desirability of0.793.The regression equation represents an empirical relation between the responses and the independent variables, here Y_4, Y_5 and Y_6 represent the protein content, TPC and FRAP content of core and X_1, X_2 and X_3 are the actual values of the independent variables.

$Y_4 = 3231.25 + 53.8704X_1 + 195.374\ X_2 - 65.0121\ X_3 - 138.012\ X_1X_2 - 337.983\ X_1X_3 - 237.196X_2X_3 + 119.672\ X_1^2 - 279.263\ X_2^2 + 63.8488\ X_3^2$ ..... (4)

$Y_5 = 25998.1 + 924.815X_1 + 23870.6\ X_2 - 29568.8\ X_3 - 188.197\ X_1X_2 - 5929.78\ X_1X_3 - 58275.2X_2X_3 - 22133.5\ X_1^2 + 17875.7\ X_2^2 + 21161\ X_3^2$ ..... (5)

$Y_6 = 195.99 - 10.8237\ X_1 + 9.2375\ X_2 + 15.1217\ X_3 + 5.66275\ X_1X_2 - 17.82X_1X_3 - 27.6583X_2X3 + 15.3883\ X_1^2 - 4.71788X_2^2 - 1.90667\ X_3^2$ ..... (6)

a b c

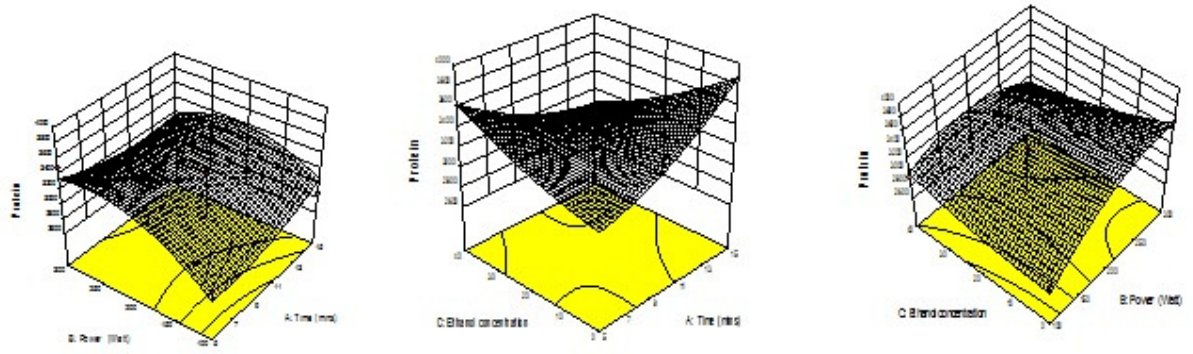

Fig 4: Response surface graphs showing effect of time(min), power(Watt) and ethanol concentration (%) on protein content(µg/g of crude extract) from pineapple core a.) Time & power b.) Ethanol concentration & time c.)Ethanol concentration & power

a b c

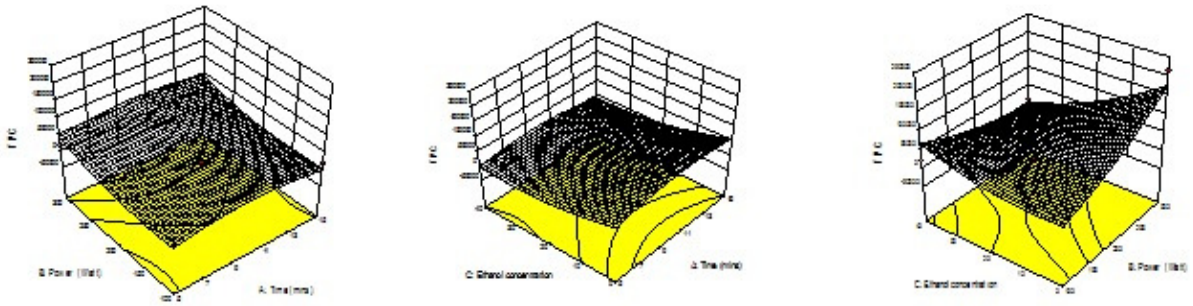

Fig 5: Response surface graphs showing effect of time (min), power (Watt) and ethanol concentration (%) on phenolic content(µg of GAE/g)from pineapple core using MAE treatment a.) Time & power b.) Ethanol concentration & time c.) Ethanol concentration & power.

a. b. c.

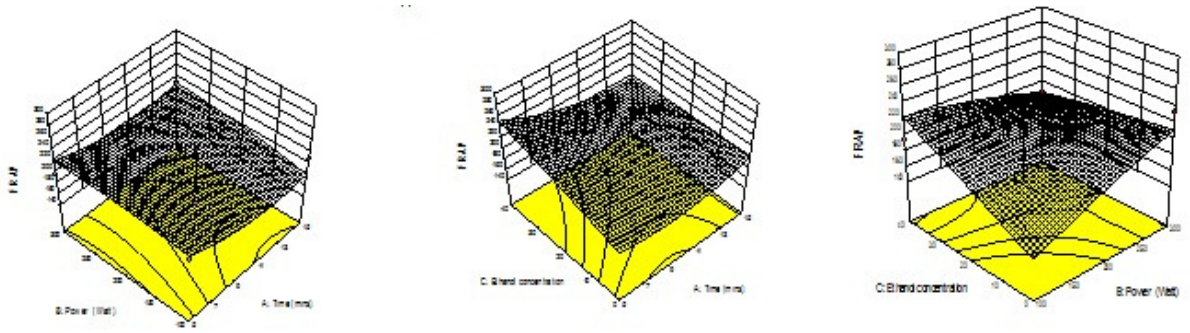

Fig 6: Response surface graphs showing effect of time(min), amplitude(%) and ethanol concentration(%) on antioxidant content from pineapple core using MAE treatment a.) Time & power b.) Ethanol concentration & time c.) Ethanol concentration & power

Determination of Bromelain activity:

The bromelain activity depends on the extraction buffer, temperature and the purification techniques used, from the study of (Chaurasiya & Umesh Hebbar, 2013). From our study the protein from the peel was found to be higher than the core, so is the bromelain activity as depicted. The protein taken was obtained as of the optimized condition and was found to be 4.903 ± 0.060 mg/ml for peel and 3.886± 0.001mg/ml for the core, therefore bromelain activity was found to be 93.61 ± 0.025 unit/ml and 92.92 ± 0.03 unit/ml respectively whereas after purification with acetone 100 % the protein content, bromelain activity and purification fold was found to 5.103 ± 0.02 mg/ml and 4.640 ± 0.001mg/ml, 120.08 ±0.02 and 111.01 ±0.05 units/ml and 1.23 and 1.00 respectively.

Specific Activity: total enzyme activity/ total protein.

Fold Purification: Specific activity after purification/ initial specific activity after purification

Protein pattern

The molecular weight of the protein of the crude waste mixture which contains (57 % peel, 28 % crown and 15 % core) has between 11.7 and 26.9 kDa. (Nor, Ramchandran, Duke, & Vasiljevic, 2015). The protein pattern of the peel from different cultivars ranged from 23- 28 kDa. (Ketnawa, 2011). Therefore the activity staining for the proteolytic activity was positive and depicted that that bromelain is found to be in the sample and found to be about 23 kDa.

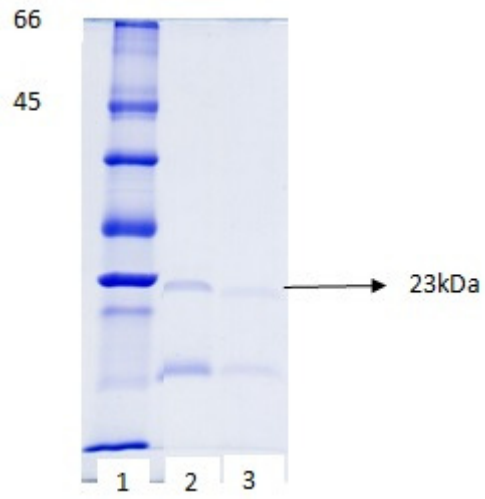

Fig 7: SDS page analysis: Lane 1 – Ladder; Lane 2- peel; Lane 3- core

Conclusion

Based on the results it can be concluded that, the optimized condition for the extraction of bioactives from peel is time 13 min, Power 252 Watt and ethanol concentration 40% yielded 4903.14 (µg/g)8273.17 (µg GAE/g) and 1090.28 µmol Fe(II)/g with a desirability 0.825, while the core maximised yield of protein 3886.92µg/g161281.21µg GAE/g 239.18µmol Fe(II)/g with the desirability 0.793. The peel contained higher protein and antioxidant power while the core had higher phenolic content. The bromelain activity was also found to be higher for the peel with a purification fold of 1.23. The obtained protein pattern was around 23 KDa for both peel and core. Microwave technique was an efficient method because of its extraction capacity, lower energy consumption, low amount of solvent, higher yield and less capital input than other conventional technquies.

Further Recommendations: The extracted bromelain can be used in various applications such as meat tenderisation, baking industry, encapsulated drugs.

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