Extraction of Proteins from Rice Mill Industry Waste using Ultrasound: It's Kinetics and Functional Properties

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Abstract : An outer layer of rice seeds is the waste for Rice Mill industry. It comprises a large number of value-added products such as Proteins (12-18 %), Fats (15-20%), Carbohydrates and various enzymes. Ultrasound-assisted extraction (UAE) of nutrition grade Protein called as Rice Bran Protein (RBP) from defatted rice bran (DFRB) was investigated and various operating parameters were optimized in the present study. To avoid denaturation and generation of impurities in the Protein isolate, extraction was performed at lower pH 9, though the extraction yield was higher at pH 11. Approximately 79% extraction was obtained by an ultrasound extraction process in 21 min as compared to 68% by the conventional process after 2 h. Final concentrate of RBP contained 79% Protein, 10.8% moisture and 2% Ash. It's functional properties such as Protein solubility index, water and oil holding capacity (3.30±0.06 and 2.76±0.01, respectively), emulsion stability and activity (31.74±0.11 and 0.59±0.05) as well as Protein digestibility (86%) were excellent. The kinetics of extraction was studied by Peleg's model, which showed good agreement with all experimental results. The results indicated an efficient application of UAE for the extraction of RBP.

Key Words : Rice Bran, Ultrasound, Extraction Optimization, Kinetic Model, RBP Composition analysis.

I. INTRODUCTION

An outer layer of rice seeds (rice bran) is an important by-product of the milling process amounting to almost about 8% of the milled rice (Shih and Champage, 1999). It has very high nutritional value as it contains 12-18% Protein (Saunders, 1990). Though it's pharmaceutical and nutritional potential is known, rice bran Protein concentrates and isolates are not available commercially. Although different methods have been reported for the extraction of Rice Bran Protein (RBP) from Defatted Rice Bran (DFRB), alkali and salt assisted extraction is the most common extraction method which gives higher yield (72%) of the Protein (AbayomiO et al., 2007; Jiamyangyuen et al., 2005;). The preparation of Protein concentration by enzymatic methods showed better results in which Protein quality was also preserved (Wasinee et al., 2008). The yield of rice bran Protein isolates increased from 34% to 76% through the use of the Phytase and Xylanase enzymes.

A group of scientists also worked on the extraction of Protein using subcritical Water and it was established that even if the process gives good yield but it is not economically feasible (Hata et al., 2008 and Pourali et al., 2009). By this method, the amount of Protein and Amino Acid produced was more eminent than those received by conventional alkali hydrolysis (Hata et al., 2008). It indicated that the subcritical Water could be employed to potentially hydrolyse defatted rice bran into the more valuable product (Pourali et al., 2009). Khan et al., 2011 and Phongthai et al., 2016 reported the microwave-assisted extraction of Protein isolates from the stabilized rice bran. It has been revealed that microwave rice bran Protein isolates had better Protein content as compared to dry heat rice bran Protein isolates. Another novel techniques such as sonication (Chittapalo et al., 2009) and sonication coupled with an autoclave (Izzah et al., 2015) were reported to improve the extraction of rice bran Protein. However, the details regarding the quality aspect of Protein, especially taste and colour have not been reported earlier.

Conventional extraction process faces the problem of lower extraction yield and high time requirements. Ultrasound-assisted extraction (UAE) found an emerging tool for extraction for better mass transfer and increased yields in many solid-liquid extraction process. UAE increases the rate of extraction and yield due to the phenomenon called acoustic cavitation. The acoustic cavitation enhances the formation, growth and collapsing of gas–vapor-filled bubbles in a liquid medium. Also, cavitation produces physical effects such as liquid circulation currents and turbulence which causes an increase in the mass transfer rates leading to increase in the extraction yield. Apart from cavitation, ultrasound-assisted extraction is also responsible for mechanical and thermal effects causing disruption of cell walls, reduction in particle size, and enhanced mass transfer across cell membranes. The mechanical effects of ultrasound can also increase the contact surface area between solid and liquid phases due to the possibility of size reduction in the solid matrix.

Although several reports are available on UAE natural compounds (Chittapalo et al., 2009). However, there is no report on modelling of extraction kinetics of RBP from DFRB. The detailed studies on colour, taste and validation of the kinetic modelling of the UAE for RBP from DFRB are yet to be investigated. Thus, this investigation was undertaken to explore the possibility of developing a technology for the extraction of RBP directly from DFRB. The objective of the present work was to maximize extraction of functional (non-denatured) Protein and with required functional properties such as Protein solubility index, water and oil-holding capacity, emulsion stability, emulsion activity and Protein digestibility. Also, Peleg's model was validated based on the experimental data for the various parameters of the ultrasound-assisted extraction of the RBP from the DFRB.

II. MATERIALS

The Rice Mill Industry Waste was obtained from the mill located in Igatpuri, Nashik (India). Experiments were performed by using analytical grade chemicals. Hexane, Sodium hydroxide, Hydrochloric acid, Coomassie brilliant blue G-250 and Ethanol, were purchased from SD fine chemicals Ltd. (SDFCL). Tris (hydroxyl methyl) methyl amine and Sodium Bicarbonate were purchased from Thomas Baker. Instruments for treatment and analysis used were spectrophotometer (Chemito spectroscan UV 2700 double beam visible spectrophotometer), Centrifuge (Remi- revolutionary high-speed centrifuge), Rotavac (Vapour, Equitron Rotevac, Medica Intsru. Mfg. Co.). Protein concentrate dried using the freeze dryer (Labconco stoppering tray dryer).
Dual frequency ultrasound cleaning bath (Model 6.5l200 H, Dakshin, India) has been used to carry out UAE with following specifications, internal dimensions 230 mm × 1500 mm × 150 mm and tank capacity of 6.5 approximately, with an ultrasonic power of 200W and frequencies of 25 kHz and 40 kHz, equipped with heater and digital temperature controller/indicator. Ultrasound bath was also equipped with 4 transducers at the base of the bath and they are mounted in zigzag position with respect to each other. Power variation is possible by varying input AC voltage through an auto - transformer. To select one operating frequency at a time a selector switch is provided on the panel.

III. METHODS

Ultrasound-assisted Extraction (UAE):
The extraction of RBP was carried out in a 100 mL flat bottom glass reactor having an internal diameter of 4.5 cm with height 10 cm. The reactor was kept 2.5 cm above from the bottom of an ultrasound bath in the axial direction. A measured quantity of the DFRB was taken in the 40 mL of buffer (Tris-HCl) having pH 8. The mixture was treated with ultrasound for 21 min. A very small amount of samples were drawn after every 3 min. All the samples were centrifuged at 8000 RPM on the micro centrifuge and diluted with DI water. The percentage of Protein extraction was analyzed by the Bradford Protein assay at 595 nm. The effect of pH (7-12), solute to solvent ratio (1:10 to 1:60), extraction time, bath temperature (30-60 °C), ultrasound frequency (i.e. 25 kHz and 40 kHz), ultrasound power (100 to 200 W) and duty cycle (25,50,75 and 100%) on RBP extraction was studied.
Batch Extraction:
Batch alkaline experiments were performed in a similar reactor used for an ultrasound with an additional assembly such as Pitched blade stirrer for agitation. Defatted rice bran (1 g) was dispersed in the buffer pH-9 solution (50 mL) with a sample to a solvent ratio of 1:50. The resulting mixture was stirred at 400 RPM for 2 h at room temperature (30±2°C). A sample was taken after every 15 min and analyzed by Bradford Protein assay.
Kinetic Model:
Jokic et al., 2008 have modelled the extraction of total Polyphenols from Soybeans using the Peleg's model. The same model was applied to predict the extraction rate constant, initial extraction rate and equilibrium concentration.
Proximate analysis:
The total Nitrogen content of DFRB was determined by the Micro Kjeldahl method. Total Protein content was estimated by using the factor 6.25. Calculated Protein content was assumed to be actual Protein content present in the DFRB. The Composition analysis of DFRB and RBP was determined by (AOAC, 2000) standard protocols. Sugar content was determined by the standard DNSA method. Total Ash content was determined by the burning the sample in the furnace at 650 °C. Oil content was determined by the Soxhlet extraction method. Fiber content was determined by acid-base treatment followed by burning at high temperature. Total Carbohydrate content was determined by the Phenol-Sulphuric Acid method. The extent of Protein was determined by Kjeldahl methods.
Statistical analysis:
All experimental results were performed in triplicate (nP3) and the data are expressed as means ±SD. Single factor ANOVA was utilized for statistical analysis. Analysis of variation is the method employed to test the equivalence of two or more population means by examining the variation of the sample that is taken. The statistical significances of process parameters were evaluated by analysis of variance (ANOVA) using Microsoft excel®. P-Value less than 0.05 ($P<0.05$) was considered to be statistically significant.
Scanning Electron Microscopy:
Three samples were prepared to compare the effect of the extraction process. The first sample witnessed for rice bran without treatment, the second one from the conventional batch extraction method for 2 h and third from UAE with the optimized conditions after 21 min. These samples were directly coated with platinum before being observed by scanning electron microscopy, scans at 5 kV at a magnification of ×500.
Functional Properties of Proteins:
Protein solubility index was determined at various pH. In order to determine the stability 20 mg freeze-dried RBP was added to 10 mL DI water and pH were adjusted in the range 1 to 11 with 0.1 N HCl and 0.1 N NaOH cautiously and stirred on a magnetic stirrer (Remi, 1MLH magnetic stirrer, India) for 1 h. Resultant solutions were centrifuged 8000 ×g and supernatants were analysed by Bradford assay to calculate the Protein solubility in mg.
Protein digestion analysis was carried out according to the method developed by Lazo et al. 1998, where 5 mL of (1.5 mg/mL) trypsin solution was taken in 5 mL Protein solution (1 mg/mL) and the temperature was maintained at 37°C. Change in pH (ΔpH) was noted after every 1 min till 10 min. Casein was taken as reference Protein. % digestivity was calculated according to formula,

$$\frac{\Delta pH\ sample}{\Delta pH\ Casein} \times 100 = \%\ Digestivity \qquad \ldots(1)$$

1% Protein solution was added to sunflower oil in a ratio 3:1 and homogenized at a speed of 15000 rpm for 1 min. Samples of the emulsion (50 µL) were taken from the bottom phase of the container at 0 and 10 min after homogenization and diluted 100 fold into 0.1% SDS solution. Absorbance was measured at 500 nm. The absorbance measured immediately after homogenization (A0) and (A10) after 10 min emulsion formation were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as shown,

$$EAI\ \left(m^2/g\right) = \frac{2\times 2.303\times A_{500}}{\emptyset\times C} \qquad \ldots(2)$$

$$ESI\ (\min) = A_0 \times \Delta_t / \Delta_A \quad \ldots(3)$$

Where,

$$\Delta_A = A_0 - A_{10} \quad \ldots(4)$$

$$\emptyset = \frac{w_d - (E \times w_1)}{w_d + w_1[[(1+E) \times D_0/D_m] - E]} \quad \ldots(5)$$

Where,

$\emptyset$ = Oil fraction
W_d = Dried weight of emulsion on heating/weight of emulsion
W_1 = Loss of weight of emulsion on heating/ weight of emulsion
D_o = Density of oil
D_m = Density of Protein solution
E = Concentration of Protein (mass per unit mass of solvent)
C = Weight of Protein per unit volume of aqueous phase.

Water and oil holding a capacity of RBP were determined according to Wani et al., 2008, RBP 100 mg was dispersed into 3 mL DI water/ oil. The mixture was homogenized using cyclo mixer (Remi India) for 5 times at 10 min interval. The suspension was then centrifuged; the supernatant was discarded and the content of residue was allowed to drain for 20 min and the gain in weight of the sample is calculated.

IV. Results and Discussion

Kinetic Study

The kinetic study of UAE of RBP from DFRB was carried out with a pH range of 7-12. The operation parameters such as solute to solvent ratio, ultrasonic power, temperature, frequency and duty cycle were studied. Figure 1a shows the graph of concentration of DFRB with respect to extraction time for different pH. It can be seen from Figure 1a that, at the initial extraction stage the mass transfer was high up to 6 min. This was due to its extracellular presence and damaged cell material which was easily pushed out of the cell with solvent. In the later stage, the extraction rate was found to be increased gradually up to 18 min. Later the extraction rate lowers down due to the diffusion resistance of a solvent through the cell wall.

Kinetic Model

The solid-liquid extraction process was carried out with a descriptive kinetic model. Different mathematical models were proposed to describe the extraction of value-added compounds from natural material.

The mathematical model proposed by Peleg's was adapted for extraction which is stated as,

$$Y(t) = \frac{t}{k1 + (k2\ X\ t)} \quad \ldots(6)$$

Where,

Y = Yield of total RBP at time t (mg/g of DFRB)
t = extraction time (min)
k_1 = Peleg's rate constant (min g/mg)
k_2 = Peleg's capacity constant (g/mg)

Y(t) can be specified by the above equation (1) by calculating k_1 and k_2 value by plotting the graph of 1/Y (t) vs. 1/t. RMSD was calculated for the model fittings for various parameters which are stated as,

$$RMSD = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(Experimental - Calculated)^2} \quad \ldots(7)$$

Optimization of process parameter and validation of model

Effect of different process parameters i.e., extraction pH (7 to 12), solute to solvent ratio (1:10, 1:20, 1:30, 1:40, 1:50 and 1:60), extraction temperature (30, 40, 50 and 60 °C), sonication power (100, 120, 150, 180 and 200 W) and sonication frequency (25 and 40 kHz) on the extraction of the RBP from DFRB were studied to optimize Protein extraction.

Influence of pH on Protein extraction

The solubility of RBP at different pH of the solvent was studied. 0.1 M Tris (hydroxyl methyl) amino methane and 0.1 M HCl buffer solution used for pH range (7-10) and for a higher pH range (11 and 12), 0.05 M $NaHCO_3$ and 0.1 N NaOH were used. In literature, RB Protein extraction was carried out using NaOH solution at different pH. Several phenolic compounds are present in RB (Tian et al., 2004), which causes deflection while adjusting the pH and also results to change in pH of the solution. However pH is a very significant factor for scale-up of the process. To maintain the pH of the solution while extraction process, instead of simple NaOH, buffers were used. Figure 1a shows, at higher pH the extraction yield increases tremendously. This may be attributed to the ultrasound treatment which enhanced the mass transfer and resulted in an increase in the rate of extraction additionally, solubility of Albumin and Globulin is higher in pH range 8-9 (Srivastava and Roy, 2011). However, at pH-11, extraction of Protein is higher in the case of rice bran. At higher pH, degradation of Protein occurs which then contributes to undesirable molecular cross-linking and rearrangement that decreases the nutritional value (Kinsella et al., 1981).

To avoid degradation, undesirable molecular rearrangement and cross-linking, experiments have been carried out at pH 9. This phenomenon is clearly seen in the Figure 2 in which, all the isolates of different pH were cooled at 0 to 4°C. At lower temperature solubility of the RBP is decreased and it settle down to the bottom of the sample bottle. Colour difference was observed at extraction pH 9, 10 and 11. Although the precipitate is higher at pH-11 but the colour of the Protein isolates is dark due to co-extraction of phenolic compound and rearrangement (Pelegrine and Gomes 2008). At pH-9 precipitate is comparably lower than pH-11 but the quality of the RBP is higher. It was also observed that at high pH denaturation of Protein occurred, which was responsible for lower yield at pH-12. Thus, pH-9 was selected as optimized value for RBP extraction. Annexure

Table. 1 shows that experimental value shows good agreement with the calculated value by solving the Peleg's mathematical equation. Both the values are plotted with 3-4% error bars in Figure 1a.

Influence of solute to solvent ratio on Protein extraction

Solute to solvent ratio plays a significant effect on the extraction. This can be attributed to the easy availability of the solvent for extracting more Protein molecules from the soild phase i.e. rice bran as well as higher volume of solvent available for enhanced solubility. Figure 1b displays the concentration profile of RBP with respect to time. It can be seen from Figure 1b that initially with 1:10 ratio, the yield was very poor. This is because of the limited availability of the solvent for the extraction. The increment in solvent ratio increased the availability of the solvent for the extraction. RBP Extraction increased with the increase in DFRB to buffer ratio (Figure 1b). At ratio 1:50 the extraction was maximu, and after that at higher ratio i.e. 1:60 it did not increased much. Figure 1b Peleg's model shows the better agreement with mathematically calculated value and experimental results. Annexure Table. 2 demonstrates the results of different solute to solvent ratio on the kinetic parameters and comparison of the experimental and calculated Yeq. It can be determined from the Annexure Table. 2 that lower RMSD as a sign of good fitting of experimental information in predicting model. So, ratio of 1:50 was finalized for extraction process.

Influence of temperature on Protein extraction

It was found that the extraction rate and yield increased with an increase in the temperature of the process. This is due to the high solubility of the solute in a solvent at a higher temperature. In the present study, the results seem to contradict earlier statements. Figure 1c indicated the yield profile of RBP with respect to time. It indicated that the extraction was higher at 40°C operating temperature, this is due to, at the low-temperature limited bubbles are formed, but they collapse with a comparatively high intensity which enhances the cell disruption. At a temperature around 30-32°C, the extraction is relatively depressed. This may be due to the lower solubility of RBP at room temperature (30-32°C). Also, the extraction decreased with a temperature above 40°C. The diminution in the collapse intensity with an increment in temperature is mainly on account of increase in the vapour pressure. Vapour pressure of the solvent increases with an increase in the temperature and more solvent vapour fill the cavitation bubbles which collapse with less intensity resulting in a reduction in cavitation. At higher temperature, surface tension is also decreased, which affect the bubble formation and collapse (Zhang et al., 2008). Thus, at higher temperature bubbles collapse with less intensity leading to decreased mass transfer rate.

On, the other hand, at higher temperature the Proteins forms an aggregates. The conformational stability of Proteins depends on upon stabilizing forces arising from a large number of weak interactions which are opposed by an almost equally large destabilizing force due mostly to conformational entropy. The value of extraction of RBP at a different time for different temperature were predicted and depicted in Figure 1c. Annexure Table. 3 showed that experimental results were matched with calculated value with a good agreement. Energy use can be ascertained by comparing the activation energy needed for ultrasound and conventional process using a Peleg's model parameter, the temperature dependence of the initial extraction rate ($1/k_1$) (1/Peleg's rate constant) is presented by the linearized Arrhenius equation giving the relation between $1/k_1$ and extraction temperature as follows,

$$ln\left(\frac{1}{k1}\right) = \ln(A) - \frac{Ea}{RT} \quad \ldots(8)$$

Where,

k_1 = Peleg's rate Constant (min g/mg RBP),
A = Constant – Frequency factor (min^{-1})
Ea = Activation Energy (J/mol)
R = Universal gas constant (8.314 J / (mol K)
T = Absolute Temperature (K)

The plot of the $\ln(1/K_1)$ vs. 1/T would result in a straight line with the negative of the slope equal Ea/R and intercept equal ln(A).

Influence of ultrasound power on Protein extraction

Cavitation generated using ultrasound is known to produce physical effects such as liquid circulation currents and turbulence which can lead to a significant increase in the mass transfer rates. The influence of ultrasound power has been studied on the extraction of RBP by changing rated power from 100, 120, 150, 180 and 200 W at frequency 25 kHz and keeping other experimental parameters constant. Figure 1d shows the yield of RBP with respect to time for different irradiation powers. It can be seen from Figure 1d that the extraction yield increased from 72.53 mg/g to 133.25 mg/g of DFRB ($P<0.05$) by increasing the ultrasonic power from 100 to 200 W for extraction time 21 min. At higher power, larger amplitude ultrasound wave generated through solvent results in the formation of a number of bubbles and collapses rapidly. The calorimetric study has been carried out to evaluated efficiency of the ultrasonic bath. Different factors like solvent, surface tension, viscosity and overall vessel geometry affect the power dissipation. Dissipated power was computed from the temperature rise of the solvent (water) after specific time at 25 and 40 kHz frequency with varying irradiated power (50 to 200 W). Practical dissipated power was found to be 40.5, 47.8, 63.2 and 69.4 W for following input power of 50, 100, 150 and 200 W respectively. However, due to limitations of the ultrasonic bath we could not perform the extraction of RBP at higher power. Thus, the ultrasonic power of 200 W is chosen as the input power for UAE of RBP. Experimental values are compared with the estimated model's value shown in Annexure Table. 4. Model values show good agreement with the experimental values plotted in Figure 1d.

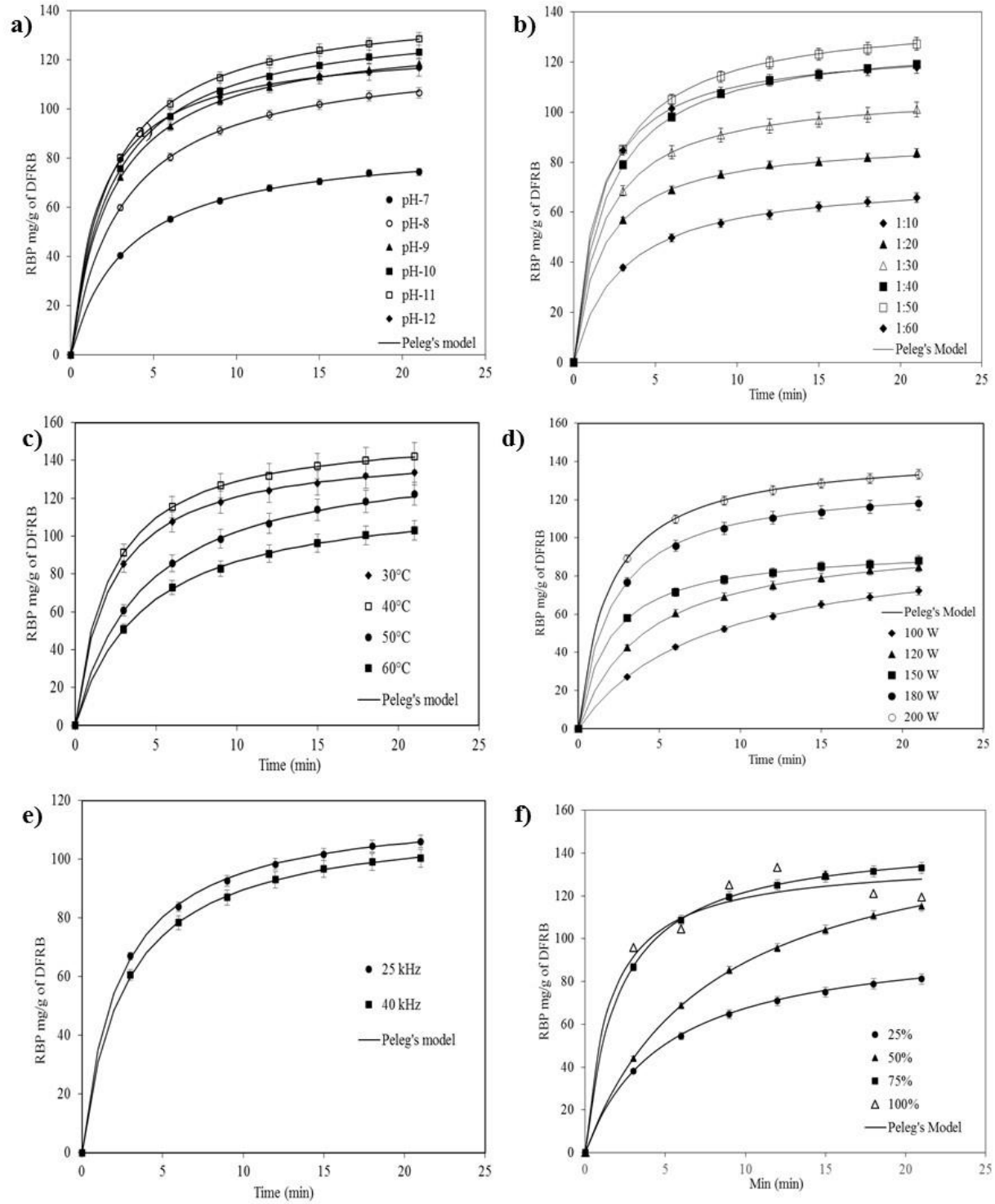


Figure 1: Influence of the a) pH, b) Rice bran to buffer ratio, c) Temperature, d) Ultrasound power input, e) Ultrasound frequency and f) Sonication duty on RBP extraction

Influence of ultrasound frequency on Protein extraction

RBP extraction was carried out with two frequencies i.e. 25 kHz and 40 kHz. Figure 1e shows the concentration of DFRB with respect to time for different operating frequency. It can be seen from Figure 1e that the extraction rate and yield are relatively higher at 25 kHz as compared to 40 kHz frequency at the same power input. Actual power dissipation is relatively lower in the case of 40 kHz than 25 kHz which was 63.7 W and 69.4 W respectively at 200 W input. Better results were observed in the case of lower frequency. At lower frequency scattering is minimum and propagation of the sound wave is proper, which allows easy cavitation. Annexure Table. 5 shows the effect of frequency on the kinetic parameters and comparison of experimental and calculated Yeq. It can be seen from the Annexure Table. 5 that the lower RMSD as a sign of good fitting of experimental data in the predicted model.

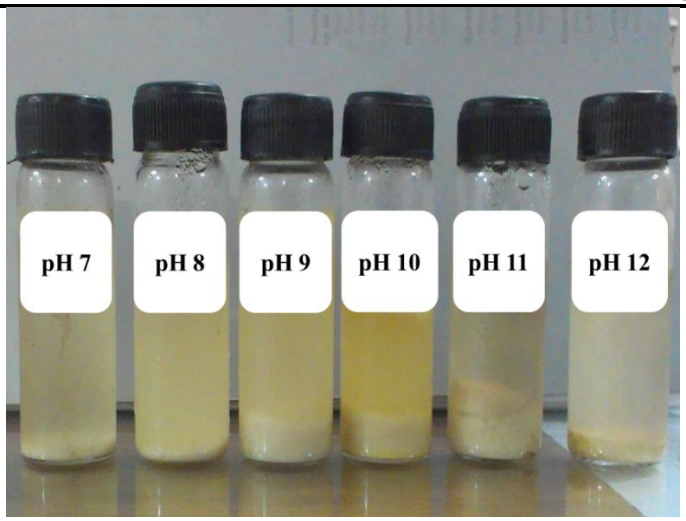


Figure 2: Effect of pH on color and extraction of RBP

Influence of duty cycle on Protein extraction

Duty cycle indicates the actual on and off time of the ultrasound. High exposer of ultrasound to the extraction mixture leads to maximum extraction. Transducer located in the base of sonication bath generates the ultrasonic wave. Long-running time of the transducer may corrode it and also excessive heat may be generated. Therefore power supply should be provided in intervals of time. Figure 1f shows the yield of RBP with respect to time for duty cycle. It can be seen from Figure 1f that the extraction is high for 75% duty cycle. It is also observed that nearly 133 mg/g of DFRB ($P<0.05$) is extracted with 75% duty cycle in 21 min. The difference between extraction yields of 100% duty cycle and 75% duty cycle is marginal; therefore 75% duty cycle was selected for the further study. Predicted model value is plotted against the experimental value in figure 1 f, which shows good agreements except for experimental values of 100% duty cycle. It is due to long time exposure to the ultrasound, which degrades RBP. Degradation of RBP affects the model fitting giving a higher value of RMSD shown as represented in Annexure Table. 6. As 100% duty cycle shows the higher extraction in lesser time, but after some time interval it showed a decrement in the extraction yield. Higher time exposer of the ultrasound to the RBP leads to more bubble formation and implosion and thus higher extraction rates are obtained. Similar to this it is possible that degradation occurs due to longer exposer of the ultrasound to the RBP. Hence the Peleg's model does not apply for 100% duty cycle because of its degradation after 12 minutes.

Comparison of the RBP extracted using UAE and conventional batch extraction

Figure 3 indicated the comparison of the RBP extracted using UAE and conventional batch extraction. It can be noted from the graph that the UAE method proves to be better than batch extraction for the RBP under similar operating parameters. Also, the time requirement for the extraction of RBP with UAE and batch extraction were 21 and 120 min respectively. Due to cavitation effect, the cells of RB was getting rupture which is resulting in higher extraction in lesser time. Hence, UAE can be employed for faster extraction of RBP over conventional batch extraction.

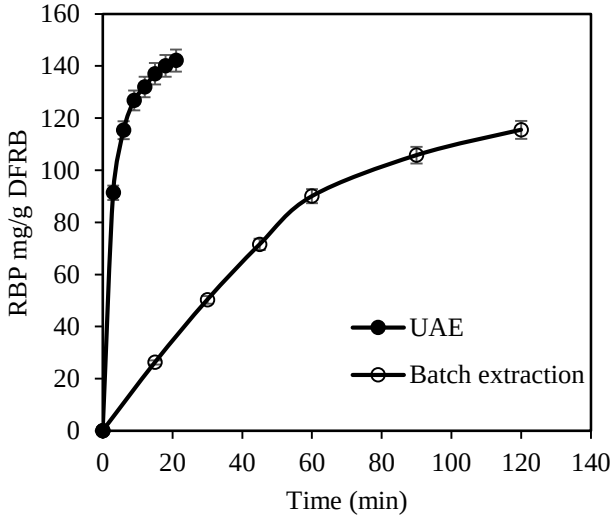
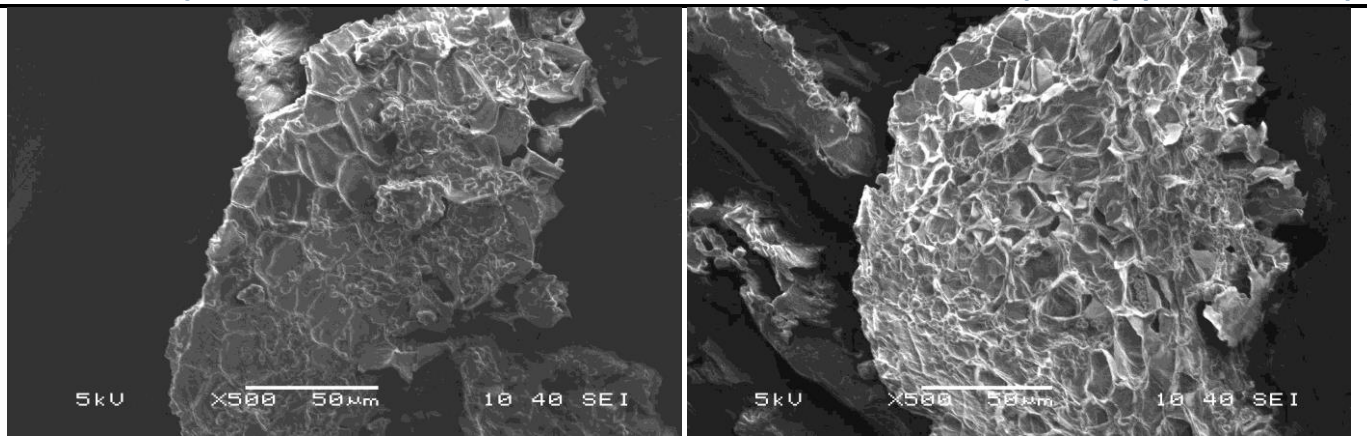


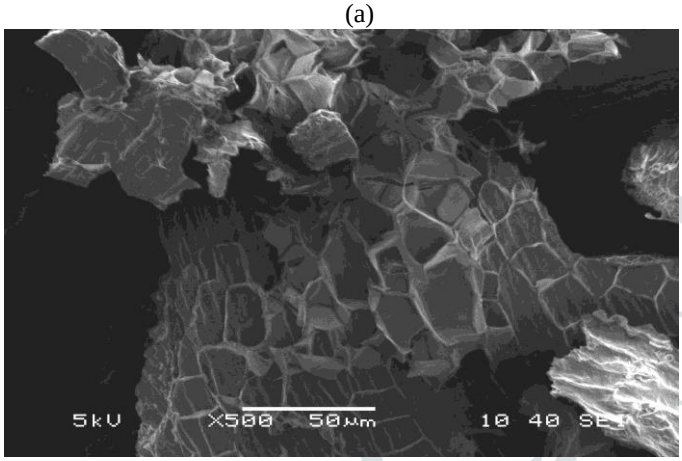
Figure 3: Comparison of Conventional batch extraction with UAE of RBP from DFRB

Effect of Ultrasound on the cell morphology

Figure 4 is the SEM image of the rice bran at a magnification 500. RB prepared from both conventional and UAE processes observed and compared with untreated RB. It can be seen untreated RB from Figure 4a the cell surface morphology is clear and uniform. In the conventional process, the contact time of an alkaline solution to RB was longer, which leads to the permeability of the cell wall to the solvent. It results in swelling of the cell and further it gets busted and extraction occurs. After drying of the residual material of conventional extraction process the rice bran cells get shrunk, it can be seen in the image Figure 4b. The effect of the cavitation on cell structure was observed in the rice bran prepared by the UAE method. Figure 4c displays breaking of the rice bran cells due to the cavitation bubbles collapsing on the surface rice bran. Micro-jets forms due to the collapsing of cavitation bubble which directed towards the cell surface, it results in the breaking of the rice bran cells and releases the material in the solvent (Gerrard et al., 2012).



(a) (b)



(c)

Figure 4: SEM images at magnification ×500 a) Untreated RB, b) Conventional batch extracted RBP and c) UA Extracted RBP

Comparative composition analysis of DFRB, RBP extracted at pH-11 and RBP extracted at pH-9

Table 1 shows the composition analysis of DFRB, RBP extracted at pH-11 and RBP extracted at pH-9. DFRB composed of 18 % Protein, 1.6 % oil, 5.48 % sugar, 6.4 % fiber, 9.7 % ash, 5.8 % moisture, 43.22 % carbohydrate and 4.9 % residual hexane remaining in the defatting process. RBP extracted at pH 9 is white in colour and not bitter in taste, furthermore, it does not contain oil and has a higher content of Protein (79%). Also, it comprises of 3.1 % fibre, 2 % ash, 10.3 % moisture and 5.1 % carbohydrate. RBP extracted at pH 11 composed of 0.40 % oil, 7.2 % fibre, 2.9 % ash, 11.2 % moisture and 6.20 % carbohydrate. It has a pale yellow colour and is bitter in taste with lower Protein (72%) content than RBP extracted at pH 9. It can be observed from Table 1 that the compositional characteristics of RBP extracted at pH-9 are found to be better as compared to RBP extracted at pH-11.

Table 1: Comparative composition analysis of DFRB, RBP extracted at pH-11 and RBP extracted at pH-9

Composition and properties	DFRB	RBP extracted at pH 11	RBP extracted at pH 9
Colour	-	Pale yellow	White
Taste	-	Bitter	Not bitter
Oil content (%)	1.6	0.40	-
Sugar (%)	5.48	-	-
Fibre (%)	6.4	7.2	3.1
Ash (%)	9.7	2.9	2
Protein (%)	18	72	79
Moisture (%)	5.8	11.2	10.8
Hexane (%)	4.9	-	-
Carbohydrate (%)	43.22	6.20	5.1

Functional Properties of RB Protein

The solubility of RBP showed (Figure 5) typical solubility curve for Proteins. RBP have the lowest solubility at pH range 4-6; isoelectric pH of RBP is in the range 4-5 where Proteins are in aggregated form resulting in precipitation. Above and below the isoelectric pH solubility of Proteins increases as the dominance of one charge either positive or negative charge increase, hence solubility increases. The improved solubility of RBP may be attributed to their smaller molecules formed due to cavitation effect and the newly exposed ionisable amino acids and carboxylic group (Phongthai et al., 2016). Although the maximum solubility of RBP showed at pH 11 with 96 % but the solubility difference between pH 9, 10 and 11 is not significant because extraction was carried out at pH 9. Digestibility of Protein was found to be 86%, indicating that at lower alkaline pH Proteins are not being complexed with other components like phenols, carbohydrates (Kinsella, 1981). At higher alkaline pH Proteins shows Millard reaction with carbohydrates and polyphenols which not only leads to loss of essential amino acids but also the formation of covalently bound aggregate, hence reducing the digestibility of Proteins (Gerrard et al., 2012).

As shown in Table 2 water and oil-holding capacity of ultrasound extracted RBP in the present study are 3.3 ±0.06 and 2.7±0.01 respectively; which are greater than the conventional extraction method. This might be due to ultrasonic waves which lead to the destruction of hydrophilic and electrostatic interaction between Protein, resulting in dissociation of aggregates (O'Sullivan

et al., 2015) leading to exposure of more hydrophilic and hydrophobic amino acid to water and oil respectively. In comparison with work done by Chittapalo et al., 2009, the results in present work showed better water and oil-holding capacity, emulsion activity (0.59±0.05) and emulsion stability (31.74±0.11). One of the reasons is the effect of pH as Chittapalo et al., 2009 done extraction at pH 11 and in present work extraction was carried out at pH 9, as discussed earlier at higher alkaline pH Proteins forms complex with polyphenols and carbohydrates. This decreases the flexibility of Protein hence surface activity decreases (Damodaran and Razumovsky, 1998). The power of sonication is also high which may have also contributed to enhancement in functional properties.

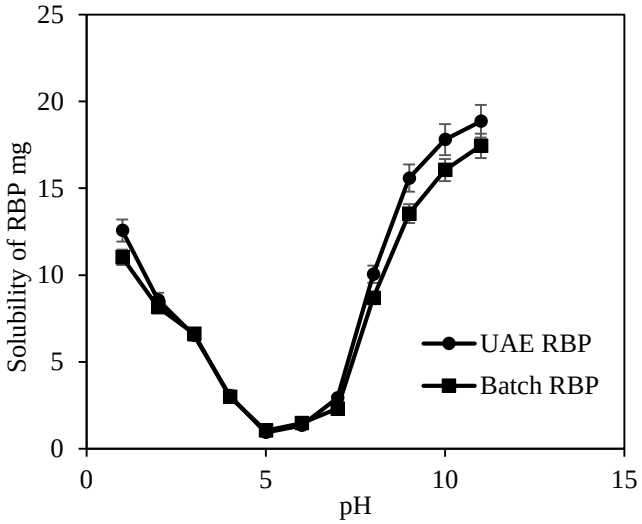


Figure 5: Solubility of UA extracted RBP and batch extracted RBP as a function of pH

Table 2: Functional properties of RBP

Functional properties	Ultrasound	Conventional	Chittapalo et al
Water holding capacity (g/g)	3.3±0.06	3.14±0.04	2.52 ±0.04
Oil holding capacity (g/g)	2.76±0.01	2.31±0.01	1.52 ±0.01
Emulsion activity	0.59±0.05	0.51±0.05	0.32 ±0.20
Emulsion stability (min)	31.74±0.11	27.03±0.10	14.36 ±0.28

V. Conclusion

Extraction of Protein from DFRB was successfully performed in presence of ultrasound. The suitable experimental parameters have been achieved with a good mathematical agreement and data fitting with the experimental results. Extraction time is lower down to 21 min from 2 h, as compared to batch extraction process is due to the mechanical effect of ultrasound wave on DFRB increases the rate of mass transfer. The mechanical effect of ultrasound wave on cell structure can be seen in SEM images. 142.088 mg/g ($P<0.05$) Protein was extracted by UAE method from the total Protein content of the DFRB which was 180.2 mg/g as estimated by the standard Kjeldahl method. The quality of the RBP with respect to its functional properties was improved as comparable with earlier reports. The Peleg's model was examined for the anticipation of the amount of RBP in the extract at a consecrated time of all extraction parameters

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VIII. ANNEXURE

Annexure Table. 1. Effect of different pH of solvent on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

pH	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g)	RMSD
7	0.0396	0.0115	74.7065	74.4	1.011
8	0.0258	0.0081	107.198	106.64	0.804
9	0.0188	0.0076	117.713	118.42	1.022
10	0.0178	0.0073	122.735	123.02	0.985
11	0.0165	0.007	128.44	128.5	0.771
12	0.0142	0.0079	116.602	116.8	1.01

Annexure Table. 2. Effect of different solute to solvent ratio on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

Solute: Solvent (Ratio)	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g)	RMSD
1:10	0.0393	0.0135	65.056	65.82	1.16
1:20	0.0193	0.0112	82.515	83.6	1.485
1:30	0.0162	0.0092	100.287	101.09	0.929
1:40	0.015	0.0077	118.840	118.98	1.072
1:50	0.0136	0.0072	127.427	127.3	1.01
1:60	0.0117	0.0079	118.243	117.9	0.954

Annexure Table. 3. Effect of temperature on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

Temperature	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g))	RMSD
30°C	0.0148	0.0068	133.24	133.56	1.061
40°C	0.0136	0.0064	141.89	142.088	0.962
50°C	0.0288	0.0069	120.89	122.31	1.89
60°C	0.0345	0.0081	102.63	103.01	1.396

Annexure Table. 4. Effect of ultrasound power on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

Power (W)	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g)	RMSD
100 W	0.0804	0.0101	71.79	72.525	0.953
120 W	0.0407	0.0099	84.47	84.8	1.146
150 W	0.0204	0.0105	87.17	88.1	1.358
180 W	0.0158	0.0077	118.309	118.025	1.645
200 W	0.013	0.0069	132.99	133.25	0.864

Annexure Table. 5. Effect of frequency on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

Frequency (kHz)	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g)	RMSD
25	0.0198	0.0085	105.9	106.02	1.51
40	0.0236	0.0088	100.76	100.3	1.06

Annexure Table. 6. Effect of duty cycle on the kinetic parameters and comparison of experimental and calculated Y_{eq} *(mg RBP/g of DFRB)*

Duty Cycle (%)	k_1	k_2	Calculated Yeq (mg RBP/g)	Experimental Yeq (mg RBP/g)	RMSD
25%	0.0491	0.0099	81.71	81.25	1.52
50%	0.0493	0.0063	115.63	115.15	0.842
75%	0.0141	0.0068	133.84	133.064	1.159
100%	0.0109	0.0073	127.89	119.646	22.279

Graphical Abstract: