Effect of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran

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Abstract

Simultaneous reduction in activity of fat destabilizing enzymes (lipase and lipoxygenase), contaminants heavy metals (As, Cd, Pb, and Hg), antinutrient phytic acid and hazardous coliforms in rice bran was investigated. Application of simple wash, soaking washed sample at different pH (2, 6 and 9) alone or in combination with ultrasonication were examined. While washing was beneficial, its low efficiency acquired further treatment, which was prevailed by application of acidic pH and ultrasound (28 kHz) treatments. Free fatty acid content and peroxide value, as indicators of enzymes activity, implied the effectiveness of treatments with adverse impact of sonication on peroxide value. Remarkably, reduction of dominant heavy metals (As, Pb and Zn) and phytic acid were synergistically facilitated by sonication. Coliforms growth was inhibited at pH 2 even at the absence of ultrasonic treatment. Therefore, combination of acidic pH and ultrasound was introduced as a practical approach to improve rice bran quality.

Keywords: Rice bran, Ultrasound, Enzyme activity, Phytic acid, Heavy metal, pH

Chemical compounds studied in this article:

sodium hydroxide (PubChem CID: 14798); hydrochloric acid (PubChem CID: 313); n-hexan (PubChem CID: 8058); soybean lipoxygenase (PubChem CID: 135321828); Tween 20 (PubChem CID: 443314); linoleic acid (PubChem CID: 5280450); toluene (PubChem CID: 1140); triacetin (PubChem CID: 5541); nitric acid (PubChem CID: 944); hydrogen peroxide (PubChem CID: 784).

1. Introduction

Rice (*Oryza sative* L.) is a staple food particularly in developing countries. Rice Bran (RB) is a by-product of milling process with an annual global production of 60 million tons (Shi et al., 2020). RB constitutes 7% ~ 8.5% of the rough rice comprising pericarp, seed coat, aleurone and sub-aleurone. Additionally, Rice Polish (RP) accounts for 2 ~ 3% of the whole grain including the inner layer of bran, germ and small portion of endosperms. During polishing step of the brown rice, RP is mainly obtained as part of RB (Malekian, 2000). Notably, it is difficult to differentiate rice polish and rice bran in a discrete term and the term "rice bran" has been used in general (Pal, 2011).

Rice bran contains 10% ~ 16% protein, 7% ~ 11.4% fiber, 15% ~ 22% lipids, 34.5% ~ 52.3% carbohydrates (Shi et al., 2020). It is also rich in vitamins E (0.32–0.44 mg/g), gamma-oryzanol (3.86–5.89 mg/g) and phenolic compounds (9.60–81.85 mg GAE/g) and other bioactive substances (S. Huang, Benchamas, & Huang, 2020). Similar to RB, RP contains a high level of protein and fat, as well as total digestive nutrients. Nevertheless, it is an important source of some essential amino acids such as lysine and threonine, it is mainly used in poultry and animal (such as ruminant and sheep) feed (Pal, 2011; Shi et al., 2020). Furthermore, there are some limitations on the application of rice milling by-products as an ingredient in human food or oil extraction. Lipid rancidity, the main source of biochemical instability of RB and RP, is induced by active enzymes including lipase and lipoxygenase which are responsible for its hydrolytic and oxidative rancidity, respectively (Monsoor & Proctor, 2002).

Another restrictive factor associated with the application of bran in food formulation is the presence of anti-nutritional phytic acid. Phytic acid is mainly localized in bran layer of rice and binds strongly with minerals cations (such as iron, calcium, zinc, magnesium and manganese). This chelating properties lead to production of phytate-mineral complexes and subsequently alters their solubility, functionality, absorption, bioavailability and digestibility (Liu, Zheng, Wang, & Chen, 2019).

Another concern deals with evidences on accumulation of toxic metals in rice and its derivatives such as oil and impose barriers on further application of RB (Liu, Zheng, & Chen, 2018; Sharafi et al., 2019).

Accordingly, there have been scientific approaches to enhance rice bran shelf life by reduction of hydrolytic and oxidative enzymes (lipase and lipoxygenase) as well as addressing mitigation in antinutrient phytic acid and hazardous heavy metals (Arsenic, Cadmium, Lead).

Several stabilization methods have been applied to inactivate the enzymes as reviewed by (S. Huang et al., 2020). Heat treatments have been the most commercial and common approach with disadvantages such as lowering the nutrient components of the bran, extensive moisture removal or incomplete and reversible inactivation of the enzymes (S. Huang et al., 2020; Malekian, 2000).

Phytic acid reduction has also been examined by processing methods such as soaking, malting, fermenting and heat treatments (Liang, Han, Nout, & Hamer, 2008; Servi, Özkaya, & Colakoglu, 2008). However, according to Servi et al. (2008) none of these processing techniques resulted in complete removal of phytic acid.

Reduction of heavy metals has also been the subject of studies, but mainly in wastewater and less attention has been paid to food by-products such as rice bran regardless of evidence on their toxic heavy metals content. Among limited studies, Sengupta et al.(2006) examined the effect of soaking and washing on rice in reducing arsenic and reported 28% reduction by sequential wash of rice in water (1:5 ratio) up to reach a clear water. Similarly, it was reported that washing significantly reduced the concentration of arsenic, cadmium and lead (Liu et al., 2018).

On the other hand, association of coliforms and bran fraction is of concern which is an indicator of fecal contamination transfer by water to rice (Lee, Park, & Ha, 2007). Skyrme, Marks, Johnson, and Siebenmorgen (1998) have reported the higher occurrence of coliforms in rough or brown rice than white rice indicating that the coliforms accumulate in bran fraction of rice.

Due to the diversity in chemical structure of RB, antinutrients and unstable components, a combined approach of physical and chemical treatments seems necessary. These processes should reduce enzymes activity, toxic heavy metals, phytic acid content and microbial growth in the final product. Ultrasound and its acoustic cavitation property as an emerging and non-thermal technology is a promising technique on enhancement of chemical treatment. Successful applications of ultrasonic on enzymes inactivation (Islam, Zhang, & Adhikari, 2014) as well as phytic acid reduction (Sivakumar, Swaminathan, & Rao, 2004) and heavy metal decontamination (Porova, Botvinnikova, Krasulya, Cherepanov, & Potoroko, 2014) have been reported previously.

In addition to physical process, chemical treatments such as change in pH are possible alternatives for enzymes inactivation. The optimum pH for lipase is around 7 and its activity is lowered by acidic solutions and alkaline treatment (Singh & Sogi, 2016). The lipoxygenase activity is reported to follow the same trend as lipase (Aanangi et al., 2016). Furthermore, phytic acid and heavy metals removal is affected by reducing pH which leads to enhanced solubility of phytic acid and heavy metals (Deng, Feng, & Qiu, 2009; Grynspan & Cheryan, 1983).

In the studies performed so far, it has been clearly pointed out that RB stabilization seems necessary in order to prolong the RB shelf life. Although, various methods have been proposed in order to stabilize RB, less attention have been paid to the combination of chemical treatment with non-thermal process such as low frequency ultrasound waves at 28 kHz to simultaneously reduce enzymatic rancidity as well as chemical and microbial barriers on application of RB as an ingredient for oil source or functional foods. Moreover, there is lack of enough information about the Solubility behavior and protein attribution of deleterious enzymes, phytic acid, heavy metals and coliforms by the application of simple wash, soaking as well as combined pH and ultrasound treatments. Generally, few studies have been performed considering reduction of toxic metals and hazardous coliforms on rice bran.

This study aims to produce an extended shelf life and safe product of RB with respect to enzymes inactivation, and contaminants and antinutrient reduction. Effects of pH and low frequency ultrasonic waves, individually or in combination, will be practiced to diminish the adverse chemical and nutritional effects of lipase and lipoxygenase, phytic acid and heavy metals including zinc (Zn), arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), as well as coliforms. Moreover, this study will re-examine the effectiveness of the treatments by evaluating the quality parameters such as free fatty acid content, peroxide value and fatty acid profile of oils extracted from RB.

2. Materials and Methods

2.1. Materials, Chemicals and reagents

Chemicals such as sodium hydroxide, hydrochloric acid, triacetin, toluene, Tween 20, acetone, diethyl ether, sodium phosphate buffer, potassium phosphate buffer, borate buffer, hydrogen peroxide (30%) and nitric acid (65%) were purchased from Merck (Darmstadt, Germany). Linolenic acid, n-hexan, thiosulphate sodium and violet red bile agar were purchased from Sigma Chemical Co. (St Louis, MO) which all were of analytical grade. Distilled water and ultra-pure water were obtained from water distiller (GFL, Type 2104, Germany). Soybean lipoxygenase (L 7395, lot 118 F03422) standard, methyl esters of the fatty acid standards and sodium salt of phytic acid (97% purity, containing 15% water) were supplied from Sigma Chemical Co. (St. Louis, USA). Standard solution of the metals (Multi_element Calibration Standard-2A for ICP) was purchased from Agilent technologies, USA.

RP was provided from the last abrasion of Hashemi variety (Shaft-Gilan Province, Iran). Due to no clear-cut definition and margin between rice bran in general and its polish segregate, the term RB is used through this manuscript. RB was passed through a sieve (750 micron aperture) in order to remove broken kernels, husk and any other foreign material. After completion, RB was stored in air tight double-layered polythene bags at -20 ^{o}C to prevent hydrolytic rancidity through lipase activity. As a result, Control rice bran (C) was prepared and used for sample preparation.

151 *2.2. Sample preparation*

152 Fig. 1 illustrates schematic view of Control rice polish (C) treated by pH and ultrasonic. To remove
153 starch from polish, a 1:5 (w/v) ratio of the C and distilled water were mixed and decanted three times
154 within 15 min followed by drying at 30 °C for 12 h using the air-ventilated oven dryer to obtain
155 Washed rice bran (W). Washed samples were adjusted to pH 2, 6 and 9 (pH–meter, Metrohm 827,
156 Swiss) and remained to be soaked for 1 h at the presence of HCl, distilled water and NaOH to obtain
157 a set of Soaked Washed Acidic (SWA), Soaked Washed Neutral (SWN) and Soaked Washed Basic
158 (SWB) bran samples. In parallel, similar pH treated samples were subjected to ultrasonic bath
159 processor (Parasonic 11s, Iran) operating at a frequency of 28 ± 5% kHz and maximum output power
160 of 150 W. This operation was conducted for 1 h with 20 min intervals at the constant temperature of
161 25 ± 1 °C (by circulating cold water in the bath). In this order, samples of Ultrasonicated Washed
162 Acidic (UWA), Ultrasonicated Washed Neutral (UWN) and Ultrasonicated Washed Basic (UWB)
163 bran samples were obtained. Both pH and ultrasonic treated samples were centrifuged (Universal 320,
164 Hettich Germany) at 9000×g for 15 min and pallets were dried at 30 °C by ventilated oven dryer to
165 reach a constant weight. This approach in sample preparation led to sets of comparable soaked and
166 ultrasonicated samples that both experienced 1 h at similar pH condition but at the presence or absence
167 of ultrasonic (Fig. 1).

168 In order to prepare RB oil samples, 200 g of each treated RB samples were transferred into a beaker
169 containing 800 mL of hexane and subjected to shake by Universal shaker (BA-SH300, Edmund
170 Buhler, Germany) for 2 h. The mixture was filtered and vacuum evaporated using rotatory evaporator
171 (HS-2005S, Hahnvapor, Korea) to get crude RB oil. To increase the efficiency of oil extraction, these
172 steps were repeated twice.

2.3. Rice bran composition analysis

Moisture (method 44–15A), ash (method 08-01), crude fat (method 30-10), protein (method 46-08), fiber (method 32-10) and starch (method 76-13) contents were determined according to AACC methods (AACC, 2000). All experiments were performed in triplicate and reported as dry weight.

2.4. Enzymes activity analysis

2.4.1. Lipase activity

Lipase activity was determined through titrimetric method as described by Hosseini, Kadivar, and Shahedi (2010) with minor modification, whereby lipase in the sample (0.5 g) hydrolyses triacetin (as a substrate) into free fatty acids during 12 h which were determined titrimetrically using NaOH (0.1 M). One unit of lipase activity was considered as the amount of enzyme which released 1mmoL of free fatty acids in 1 h under essay conditions. Final estimation were calculated according to below formula:

Lipase activity (U/g) = [(mL NaOH of sample-mL NaOH of blank) × Molarity (0.1 M)] / [Reaction time (12 h) × Weight of sample (0.5 g)]

2.4.2. Lipoxygenase activity

The assay was performed spectrophotometrically according to the method described by Tolouie, Mohammadifar, Ghomi, Yaghoubi, and Hashemi (2018) with minor modifications. The standard enzyme solution was prepared and used as a control for each analysis (Ramezanzadeh, Rao, Windhauser, Prinyawiwatkul, & Marshall, 1999). The total reaction system contained 2.4 mL of potassium phosphate buffer solution, 300 μL of the extracted enzyme and 300 μL of substrate, by which lipoxygenase oxidizes linoleic acid (as a substrate) into conjugated dienes that were determined at the beginning and after 5 min at 234 nm (25 °C) using spectrophotometer (Perkin Elmer lambda 2, USA). One unit of lipoxygenase activity was considered as the changes in absorbance of 0.001/min under indicated experimental conditions and calculated according to the following equation:

Lipoxygenase activity (U/g) = [(Changes in absorbance at 234 nm) ×1000] / [(Reaction time (5 min) × Weight of sample (1 g)]

2.5. Rice bran oil analysis

2.5.1. Rice bran oil yield

After cold extraction of rice bran oil, as mentioned in section 2.2, the yield of extracted oil was calculated according to below equation and expressed as percentage of rice bran used for oil extraction. .

Oil yield (%) = (weight of extracted oil / weight of rice bran) ×100

2.5.2. Free fatty acid content (FFA)

Free fatty acid (FFA) content as a primary index of lipase activity was determined using No. 940.28 standard method of AOAC (AOAC, 1995). FFA was measured as oleic acid and expressed as percentage of oil.

2.5.3. Peroxide value (PV)

PV, the primary indicator of lipoxygenase activity, was determined according to AOAC official method of 965.33 (AOAC, 1995) and expressed as meq O_2/kg oil.

2.5.4. Fatty acid composition

The fatty acid composition of RB oil was determined according to AOAC No. 996.06 (AOAC, 1995). The analysis of fatty acid methyl esters (FAMEs) was carried out using a gas chromatograph (GC) (Agilent Technologies, USA) coupled with a flame ionization detector (FID) and a RTX-2330 capillary column (105 m, 0.25 mm, 0.2 m of film thickness) (Agilent Technologies, USA). The temperatures of injector and detector were set at 250 and 270 °C, respectively. Hydrogen was used as a carrier gas with a flow rate of 1 mL/min. The oven temperature was maintained at 50 °C for 1 min after injection, then increased to 198 °C with the rate of 20 °C/min, which was hold for 60 min. Fatty acid identification was based on comparison with methyl esters of the fatty acid standards.

221 *2.6. Phytic acid analysis (PA)*

222 PA was determined by spectrophotometric procedure according to the method described by Vitali,
223 Dragojević, Šebečić, and Vujić (2007) based on the principle that the decrease in absorption of iron
224 content was measured at 519 nm by addition of 2.2′ biprydyl solution. Calibration curve was obtained
225 by sodium salt of phytic acid standard and used in range of 500-2000 μg/g. Phytic acid concentration
226 was expressed as g/kg RB.

227 *2.7. Heavy metals analysis*

228 Digestion of samples was conducted according to method described by Liu et al. (2018) with
229 modifications. The microwave vessels (Multiwave 3000, Rotor 8NXQ80, Anton Paar, Austria) were
230 cleaned with nitric acid followed by washing with ultra-pure water. The flasks were prepared by wash
231 with nitric acid for 30 min and rinsed with ultra-pure water. Precisely, 1 g (dry weight) of sample was
232 weighted into the flask followed by addition of 6 mL nitric acid and 2 mL of hydrogen peroxide and
233 transferred to microwave vessels. The microwave digestion was carried out by following program:
234 adjustment of power to 200 W within a ramp time of 15 min, hold for 30 min with subsequent 30 min
235 hold at the power of 400 W and the ramp time of 20 min. Final adjustment of microwave digestion
236 was set to ramp up the power to 600 W within 30 min followed by the third holding time for 30 min.
237 The mixtures were filtered by ashless filter paper (Whatman number 42) and diluted with ultra-pure
238 water to reach 10 mL. In parallel, blank test was also prepared.

239 The contents of heavy metals including Zn, As, Cd, Pb and Hg, in RB were analyzed by an Inductively
240 Coupled Plasma-Mass Spectroscopy (ICP-MS) (Agilent 7500, Agilent technologies, USA) coupled
241 to a Modified Lichte as a type of cyclonic spray chamber. The Instrumental conditions were as
242 following: Radio Frequency (RF) generator power of 1200 W, resonance RF frequency at 24 MHz,
243 plasma gas flow rate 12.2 L/min, auxiliary gas flow rate 0.8 L/min and nebulizer argon gas flow rate
244 0.8 L/min. Three replicate measurements with 250 total(s) uptake time for each. The limit of detection
245 (LOD) of the analyzer for Zn, As, Cd, Pb and Hg was 0.086, 0.006, 0.007, 0.005 and 0.033 μg/kg,

246 respectively. Meanwhile, the limit of quantitation (LOQ) for these heavy metals was in the order of
247 0.285, 0.022, 0.025, 0.016 and 0.110 μg/kg. The obtained results from ICP-MS were multiplied by
248 the dilution coefficient. The obtained results from ICP-MS were multiplied by the dilution coefficient
249 and expressed as μg/kg.

250 *2.8. Coliforms quantitative analysis*

251 Coliforms counts was performed according to the method described by Lee et al. (2007) using serial
252 dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of samples and violet red bile agar (VRBA) medium. After
253 incubation for 24 h at 37 °C, colonies were counted on colony counter (Funke Gerber, Colony Star
254 8500, Germany) and expressed as Colony Forming Units per gram (CFU/g).

255 *2.9. Statistical analysis*

256 All the experiments were performed in triplicates and data are shown as mean ± standard deviation
257 (SD). One-way Analysis of Variance (ANOVA) was performed using IBM SPSS Statistics version
258 25.0 (SPSS Institute, Chicago, IL, USA). In specific, paired sample t-test signified the effect of
259 washing in compare with control and the difference between soaked and ultrasonicated samples at
260 certain pH. To investigate the synergistic effects of pH and ultrasound, two-way ANOVA test was
261 applied. A p-value < 0.05 was considered to be statistically significant.

262 **3. Results and Discussion**

263 *3.1. Rice bran composition analysis*

264 RB was composed of moisture = 7.06 ± 0.03%, ash = 14.11 ± 0.12%, crude fat = 13.76% ± 0.08,
265 crude protein = 10.2 ± 0.27%, crude fiber = 20.91 ± 0.14% and starch = 21.65 ± 0.37%. The mean
266 values obtained in the present study is in line with Hossain et al. (2012) who reported moisture =
267 4.0% ~ 11.4%, ash = 7.1% ~ 17.6%, crude protein = 4.7% ~ 11.4%, and crude fiber = 6.4% ~ 41.5%
268 depending on the type of RB.

3.2. Enzymes activity

3.2.1. Lipase activity

Table 1 depicts reduction in lipase activity of the control by all treatments at different extent. About 50% reduction of the enzyme activity was observed after washing control rice bran sample. Water solubility behavior of lipase can account for this reduction and it is reasonable to assume that significant amount of the enzyme has been discarded along with starch during de-starching by wash. While three times water wash for 15 min reduced the enzyme activity in W, soaking the Washed sample (W) at neutral pH (SWN) for 60 min increased the activity from 0.047 ± 0.001 U/g in W to 0.068 ± 0.001 U/g for SWN. The result of this study is in line with Loikeao, Vayupharp, and Laksanalamai (2011) who also observed an increase in lipase activity of rice flour as the soaking time increased. Generally, soaking causes enhancement of water activity which leads to an increase in lipase activity as is also shown by Tolouie et al. (2018). Application of ultrasound for 60 min did not further decrease the enzymatic activity. Comparatively, reducing pH by soaking W at pH 2 to produce SWA decreased lipase activity by 24% which was 63% reduction relative to control. Enzyme denaturation at acidic pH in SWA can account for this decline. Singh and Sogi (2016) also used hydrochloric acid as a simple chemical approach for RB stabilization. They showed that lipase activity was reduced by lowering the pH. At this pH, ultrasound significantly increased the efficiency of lipase inactivation by 12% and lowered lipase activity in UWA comparing to SWA ($p < 0.05$). Investigating the effect of basic pH, indicated while soaking at pH 9 could decrease the lipase activity of the control from 0.097 ± 0.001 to 0.060 ± 0.000 U/g in SWB but compared to initial W sample increasing trend of lipase activity is noted (which also occurred at neutral pH). Considering the optimum pH of 7.5-8 for lipase, soaking close to this pH within this time could enhance the activity as mentioned by Singh and Sogi (2016). They showed that lipase is stable over the pH range from 7 to 9. Notably, ultrasonication at basic pH (UWB) for similar time as in SWB significantly increased the efficiency of lipase inactivation by around 6% emphasizing low but efficient application of ultrasonic on lipase inactivation ($p < 0.05$). Therefore, comparison the effect of pH on washed sample,

lipase showed its lowest activity at acidic pH followed by alkaline and neutral pH when the W sample was soaked for 1 h.

In addition, at similar pH condition, ultrasound increased the efficiency of lipase inactivation by 12, 2, and 6% for UWA, UWN and UWB (relative to SWA, SWN and SWB), as mentioned earlier, which were statically significant at acidic and alkaline pH. Therefore. Ultrasonic alone at neutral pH was not effective enough to reduce the enzyme activity, although the combined effect of ultrasound and pH indicated that ultrasound was able to enhance the efficiency of lipase inactivation at acidic or alkaline conditions. Generally, the effect of ultrasound on enzyme denaturation can be described through three mechanisms: Firstly, ultrasound waves cause cavitation and acoustic streaming in the vicinity of the liquid medium providing sufficient mechanical, thermal, and chemical effects to denature the enzymes (Islam et al., 2014). Secondly, sonication decompose the water molecules to form H and OH free radicals, consequently promoting chemical reactions(Islam et al., 2014). Some amino acid residues (including lysine, leucine, isoleucine, proline, and glutamic acid) contribute in enzyme stability, substrate binding and catalytic activity. They can easily react with the free radicals and subsequently affect the enzymes functions (Muthukumarappan, Tiwari, O'Donnell, & Cullen, 2016; Islam et al., 2014). Thirdly, The OH radicals can combine to produce hydrogen peroxide which is known to be an efficient inhibitor for some enzymes such as lipase or lipoxygenase (Muthukumarappan et al., 2016; Islam et al., 2014). The lower enzyme activity at lower pH in combination with ultrasound can be attributed to the development of hydroperoxy radicals (HO_2). Since pK_a of HO_2 radical is around 4.7, their formation at low pH will be accelerated. Also, this protonated superoxide go through H^+ dependent reactions to generate H_2O_2. Therefore, the H_2O_2 concentration is increased at low pH and as mentioned above, it can inhibit some enzymes activity such as lipase and lipoxygenase (Muthukumarappan et al., 2016). Additionally, two-way ANOVA indicated that pH and ultrasound did not exhibited a synergistic interaction on lipase inactivation ($p > 0.05$) as shown in supplemental Table. 5.

3.2.2. Lipoxygenase activity

Lipoxygenase activity of all treated samples were reduced compared to the control in a similar trend of lipase activity (Table 1). The initial lipoxygenase activity was reduced by 57% after de-starching by washing with water. This reduction can be explained by a wash-out effect and removal of FFA (as shown in Table 1) which are the lipoxygenase substrates (Monsoor & Proctor, 2002). Soaking the W sample at neutral pH increased the lipoxygenase activity but still in a value lower than control. Lipoxygenase could regenerate its activity after water absorption during soaking. These results are in agreement with Tolouie et al. (2018) who reported that lipoxygenase could regenerate its activity after water absorption. Similar to lipase, the effect of sonication at neutral pH was not significant, although 15% of activity reduction in UWN compared to SWN was observed. Regarding soaking at acidic pH, similar to lipase, lipoxygenase activity was reduced 28% and 70% in comparison with W and C, respectively. Interestingly, by application of ultrasonic at acidic pH, 50% more reduction of activity was observed in UWA. The lipoxygenase activity in SWB did not exhibit difference to the W even at the presence of ultrasonic treatment (UWB). Nevertheless, both treatments resulted in lowering the activity of lipoxygenase compared to control. Similarly, Aanangi et al. (2016) examined the effect of pH ranged between 3-10 on lipoxygenase activity and demonstrated its highest activity at pH 6.5 and its lowest activity at pH 3 and 10 which are in line with observed low activity of this enzyme in SWA and SWB but high activity in SWN of this study.

Overall, application of ultrasound reduced lipoxygenase activity by around 50, 15 and 14% (UWA, UWN and UWB, respectively) compared to their parallel SWA, SWN and SWB (not significant for the last two). Similar to lipase enzyme, the ultrasonicated sample at acidic pH showed the minimum activity among all samples. The effectiveness of sonication on the enzyme inactivation can be explained through several mechanisms which were all mentioned earlier for lipase (section 3.2.1). Similar to lipase, no synergistic interaction ($p > 0.05$) was observed between pH and ultrasound treatments on lipoxygenase inactivation by two-way ANOVA (Supplemental Table. 5.)

3.3. Rice bran oil analysis

3.3.1. Rice bran oil yield

As provided in Table 1, the obtained results showed that with washing of RB, the oil yield increased in comparison with untreated RB (C). The possible explanation could be due to the starch removal and soluble solid contents leaching which resulted in enhancement of oil percent of initial rice bran at the constant weight. Similarly, soaking at neutral pH known as SWN also caused an increase in oil extraction efficiency. Regarding the effect of pH, it was demonstrated that the oil yield decreased by increasing pH to 9 in SWB, which might be due to the saponification since alkali can react with FFAs affecting the quantity of .extracted oil. The achieved results are not in line with the study performed by Khoei and Chekin (2016) who investigated the effect of pH on ultrasound-assisted extraction of RB oil. Although, they showed that oil recovery was enhanced by increasing pH, they measured the oil which obtained after centrifuge by aqueous extraction. In our experiment, similarly, some amount of the oil might be lost during centrifuging of treated samples and was discarded by the supernatant. Therefore, the oil content in treated sample at basic pH (SWB) was reduced and less oil was extracted.

Sonication, as another treatment, increased the yield of oil extraction compared with non-sonicated samples (SWA, SWN and SWB). It was mentioned that ultrasound waves can facilitate mass transfer leading to enhancement of oil yield. Moreover, ultrasonic treatment provides damages on cell membrane and increase the number of holes leading to enhancement of oil yield (Moghimi, Farzaneh, & Bakhshabadi, 2018).

3.3.2. Free fatty acid content

The content of FFA, as a primary indicator of lipase activity, is shown in Table 1. Washing had positive effect on the stability of the extracted oil from RB as its content significantly declined from 2.86% in C to 2.27% in W ($p < 0.05$). Expectedly, reduction of lipase activity, which is reported in this study, induced this declining trend. Monsoor and Proctor (2002) studied the influence of water washing as a simple technique on the removal of surface total lipids and FFA on rice. They reported

that total surface lipids and FFA were reduced on rice by water washing for 5 to 10 min. The effect of soaking at neutral pH on the quality of RB oil indicated that regardless of beneficial influences of wash, soaking after wash as in SWN, exhibited detrimental effects. A possible explanation for the significantly increase in FFA from 2.27% in W to 2.71% in SWN ($p < 0.05$) is the reactivation of lipase enzyme as shown by increase in lipase activity at elevated a_w and leading to accelerate the formation of FFA. Similarly, a small increase in the FFA content of UWN was observed due to the ultrasound which reached to 2.80% in UWN. The lowest amount of FFA was observed in SWA (1.90%) and UWA (1.93%) as a result of lipase inactivation. The amounts of FFA in SWB and UWB were 2.05% and 2.13%, lower than neutral but higher than acidic condition, which followed the same pattern as lipase activity. Among samples soaked at different pH, it was expected to observe lowest FFA content of extracted oils for SWA as it demonstrated lowest lipase activity.

The comparison between ultrasonicated and non-ultrasonicated samples at each pH showed only a small and not significant increase in FFA of UWN, UWA and UWB (Table 1) due to the effect of free radicals formed during sonication which may lead to an increase in FFA as shown by Moghimi, Farzaneh, and Bakhshabadi (2018).

As mentioned by Patil, Kar, and Mohapatra (2016) RB with FFA content less than 5% and RB oil with less than 10% are proper for human consumption. Within the advancement of storage time, FFA content of W increased slightly (less than 5%) due to lower lipase activity in compare to C. Therefore, washing found to be efficient in controlling lipase activity and FFA increase until the 90 days of storage. In contrast, among soaked samples at different pH, FFA content of SWN exceeded to beyond the acceptable limit (which is < 5%) due to higher lipase activity which hydrolyze triglycerides into FFA. As mentioned, ultrasound alone was not able to reduce lipase activity at neutral pH, therefore FFA content of UWN increased drastically during the storage. The FFA content of acidic and basic samples with the presence or absence of ultrasound in SWA, UWA, SWN and UWB did not increased sharply to beyond the acceptable limit (which is < 5%) and lowest FFA was observed for SWA and UWA as the lipase showed its lowest activity at acidic conditions. As a result, the increase in FFA

content is affected by lipase activity which means acidic pH could hinder lipase activity and FFA release from triglycerides. As ultrasound could not decrease the FFA content during the initial day of storage, no significant reduction was also observed till the 90 days of storage. However, the rate of FFA formation had a small decrease in compare to their parallel soaked samples as a result of reduced lipase activity.

3.3.3. Peroxide value (PV)

PV as another important factor for oil quality is influenced by lipoxygenase activity. The PV for C and W were 11.50 and 3.65 meq O_2/kg oil, respectively, which highlights the effectiveness of wash and its relation with lipoxygenase activity (Table 1). Washing was found to be more effective on the reduction of lipoxygenase activity and PV in comparison with lipase activity and FFA formation which indicated that lipase structure has higher resistance to water wash and drainage. Soaking at different pH have negative effect on oil quality which is denoted by increase in PV from 3.65 in W sample to 9.5, 9.67 and 4.92 meq O_2/kg oil in SWN, SWB and SWA, respectively. Soaking at neutral and basic pH accelerated FFA formation which subsequently assisted oxidation by lipoxgenase and increase in PV. In addition, higher lipoxygenase activity of alkaline and neutral samples (with and without ultrasound) facilitated oxidation. In case of acidic treatment, while SWA presents PV higher than W, still it was significantly lower than SWN and SWB. It would be reasonable to assume lipoxygenase activity has been decreased at acidic pH due to protein denaturation (Aanangi et al., 2016) and consequently lower PV would be obtained compared to alkaline and neutral condition. However, regardless of lower lipoxygenase activity of SWA and UWA than W, these samples still demonstrated greater PV than W which might be due to the duration of soaking or other factors involving in PV formation.

Ultrasonication also increased the PV of these samples compared to their counterparts as in UWN, UWB and UWA (Table 1). Production of free radicals and elimination of antioxidants which are

favorable condition for higher oxidation rate have been reported previously for ultrasound pretreatment in oil extraction (Böger et al., 2018; Moghimi et al., 2018).

Noticeably, rice bran oil containing PV < 10 meq O_2/kg is considered suitable for human consumption (Patil et al., 2016). In our study, during storage time, the PV of W sample was around the acceptable limit (<10 meq O_2/kg) up to 30 days of storage, while the PV of C sample was higher than 10 meq O2/kg even at the initial day of oil extraction. As soaking at neutral pH increased the lipoxygenase activity in compare to W, the PV of SWN also increased to beyond the acceptable limit after 30 days of storage. Although ultrasound increased the PV at the initial day of storage in comparison with non-sonicated samples, no significant difference ($p > 0.05$) was observed with the advancement of storage period to 30 and 60 days. It is reasonable to assume that the PV increased at the first days of storage as a function of processing conditions and the negative effects of ultrasound on the oil quality, while during storage the formation of peroxides and hydro-peroxides followed the same rate in sonaicated and soaked samples at each pH as affected by lipoxygenase activity.

Table 1

Effect of pH and ultrasound treatments on enzymes activity, oil yield and its quality.

Parameter / Sample Type	Oil yield (%)	Lipase activity (U/g)	Free fatty acids (%Oleic acid) Storage time (days)			Lipoxygenase activity (U/g)	Peroxide value ($meqO_2/Kg$) Storage time (days)		
			0	30	60		0	30	60
Control (C)	5.65±0.30^{e}	0.0970±0.0017^{a}	2.87±0.08^{a}	31.66±1.80^{a}	54.77±1.50^{a}	2.20±0.20^{a}	11.50±0.40^{a}	37.38±1.83^{a}	67.55±1.25^{a}
Washed (W)	6.30±0.18^{d}	0.0473±0.0010^{e}	2.28±0.04^{c}	3.37±0.30^{cd}	3.78±0.10^{de}	0.93±0.21^{c}	3.65±0.20^{f}	10.76±0.12^{d}	16.19±0.73^{d}
Soaked Washed at Neutral pH (SWN)	7.13±0.10^{c}	0.0687±0.0010^{b}	2.72±0.04^{b}	18.31±0.55^{b}	30.05±1.15^{b}	1.53±0.39^{b}	9.50±0.30^{c}	24.99±1.60^{b}	33.22±0.40^{b}
Ultra-sonicated Washed at Neutral pH (UWN)	7.67±0.23^{b}	0.0667±0.0012^{b}	2.81±0.06^{ab}	17.14±0.40^{b}	30.34±0.80^{b}	1.47±0.12^{b}	11.00±0.35^{a}	25.63±1.04^{b}	33.65±0.25^{b}
Soaked Washed at Acidic pH (SWA)	7.28±0.20^{b}	0.0360±0.0017^{f}	1.91±0.03^{f}	2.17±0.45^{d}	2.71±0.20^{ef}	0.67±0.12^{d}	4.92±0.25^{e}	9.89±0.40^{de}	13.65±0.32^{e}
Ultra-sonicated Washed at Acidic pH (UWA)	8.00±0.15^{a}	0.0313± 0.0015^{g}	1.94±0.03^{ef}	2.11±0.07^{d}	2.24±0.10^{f}	0.33±0.12^{e}	7.79±0.30^{d}	7.87±0.22^{e}	12.86±0.68^{e}
Soaked Washed at Basic pH (SWB)	6.53±0.20^{d}	0.0603± 0.0006^{c}	2.06±0.04^{de}	4.64±0.20^{c}	5.33±0.30^{c}	0.93±0.23^{c}	9.67±0.4^{c}	20.97±0.65^{c}	27.38±0.53^{c}
Ultra-sonicated Washed at Basic (UWB)	7.03±0.13^{c}	0.0563± 0.0015^{d}	2.13±0.05^{d}	4.39±0.35^{c}	4.61±0.08^{cd}	0.80±0.20^{c}	10.31±0.4^{b}	21.11±1.95^{c}	26.92±0.80^{c}

*Values are mean ± standard deviation in triplicate. Samples with different letters within the same column are significantly different ($p < 0.05$).

3.3.4. Fatty acid composition

The fatty acid composition of RB oil extracted after various treatments are given in Table 2. The GC analysis showed highest polyunsaturated fatty acids content and ratio of unsaturated to saturated fatty acids for W among all other treatments. The effectiveness of washing to improve fatty acids profile can be attributed to lower lipoxygenase activity. The elevated linoleic acid content of washed rice bran oil is of great importance since it is an essential fatty acid which lowers Low Density Lipoprotein (LDL). On the other hand, lower polyunsaturated fatty acids and unsaturated to saturated fatty acids ratio for SWN relative to W was observed. Higher activity of lipoxygenase in SWN could account for this difference. The GC analysis showed no significant difference ($p > 0.05$) in the ratio of unsaturated to saturated fatty acids between SWA, SWN and SWB samples indicating that pH had no effect on fatty acid profile at mentioned pH values. However, a reduction of polyunsaturated fatty acids at basic pH in compare to acidic and neutral was observed, which may be due to saponification of the fatty acids. Singh and Sogi (2016) mentioned that high pH values may lead to deterioration of oil quality by saponification. Additionally, comparison of each soaked sample at certain pH with its sonicated counterparts revealed reduction of the polyunsaturated fatty acids to some extent due to the ultrasound as it has been reported earlier by Hernández-Santos et al. (2016). Although, no significant difference ($p > 0.05$) was observed in the ratio of unsaturated to saturated fatty acids between soaked and sonicated samples at each pH.

Table 2

Effect of pH and ultrasound treatments on fatty acid composition.

Sample type **Fatty acids**	Control (C)	Washed (W)	Soaked Washed Neutral (SWN)	Ultra-sonicated Washed Neutral (UWN)	Soaked Washed Acidic (SWA)	Ultra-sonicated Washed Acidic (UWA)	Soaked Washed Basic (SWB)	Ultra-sonicated Washed Basic (UWB)
Saturated fatty acids*								
C12:0	0.03±0.000^{c}	0.46±0.002^{b}	0.04±0.001^{bc}	0.05±0.002^{b}	0.04±0.003^{bc}	0.03±0.002^{c}	0.42±0.020^{a}	0.048±0.004^{b}
C14:0	0.49±0.02^{a}	0.48±0.01^{ab}	0.46±0.01^{b}	0.48±0.00^{ab}	0.46±0.03^{b}	0.49±0.01^{a}	0.49±0.01^{a}	0.49±0.00^{a}
C16:0	16.37±0.04^{a}	14.94±0.07^{c}	15.66±0.09^{abc}	15.90±0.06^{ab}	15.77±0.10^{ab}	15.90±0.12^{ab}	15.49±1.2^{bc}	15.73±0.08^{abc}
C18:0	2.33±0.05^{a}	2.18±0.03^{b}	2.16±0.07^{b}	2.29±0.11^{ab}	2.23±0.04^{ab}	2.23±0.06^{ab}	2.27±0.10^{ab}	2.26±0.05^{ab}
C20:0	1.08±0.03^{a}	0.95±0.05^{b}	0.98±0.07^{ab}	1.06±0.08^{a}	1.00±0.01^{ab}	0.99±0.00^{ab}	0.98±0.04^{ab}	1.05±0.08^{ab}
C22:0	0.45±0.01^{abc}	0.41±0.02^{c}	0.42±0.05^{bc}	0.48±0.01^{a}	0.46±0.01^{ab}	0.42±0.04^{bc}	0.41±0.02^{c}	0.47±0.00^{a}
C24:0	0.77±0.06^{ab}	0.63±0.02^{cd}	0.72±0.08^{abc}	0.76±0.01^{ab}	0.60±0.04^{d}	0.67±0.06^{bcd}	0.73±0.02^{abc}	0.8±0.10^{a}
SFA	**21.52±0.21^{a}**	**19.63±0.14^{c}**	**20.44±0.51^{bc}**	**21.02±0.27^{ab}**	**20.56±0.14^{abc}**	**20.73±0.29^{ab}**	**20.79±1.37^{ab}**	**20.70±0.44^{ab}**
Mono-unsaturated fatty acids*								
C16:1	0.77±0.04^{a}	0.38±0.06^{b}	0.38±0.10^{b}	0.35±0.02^{b}	0.34±0.05^{b}	0.37±0.30^{b}	0.7±0.14^{a}	0.4±0.07^{b}
C18:1	48.1±1.60^{a}	47.64±1.30^{a}	47.53±0.09^{a}	48.43±0.05^{a}	47.4±0.70^{a}	47.9±0.90^{a}	47.25±0.40^{a}	47.9±0.20^{a}
C20:1	0.67±0.2^{a}	0.62±0.03^{a}	0.63±0.01^{a}	0.66±0.07^{a}	0.65±0.04^{a}	0.63±0.08^{a}	0.59±0.07^{a}	0.65±0.03^{a}
MUFA	**49.54±1.84^{a}**	**48.64±1.39^{a}**	**48.54±0.20^{a}**	**49.44±0.14^{a}**	**48.39±0.79^{a}**	**48.9±0.95^{a}**	**48.54±0.61^{a}**	**48.96±0.28^{a}**
Poly-unsaturated fatty acids*								
C18:2	26.22±0.50^{e}	29.87±0.30^{a}	29.2±0.05^{b}	27.51±0.08^{d}	29.3±0.20^{b}	28.31±0.04^{c}	27.5±0.10^{d}	27.43±0.06^{d}
C18:3	0.91±0.02^{b}	1.13±0.10^{b}	1.08±0.12^{b}	0.98±0.05^{b}	1.1±0.03^{b}	1.02±0.03^{b}	1.75±0.07^{a}	0.98±0.04^{b}
PUFA	**27.13±0.52^{e}**	**31±0.20^{a}**	**30.28±0.07^{b}**	**28.49±0.13^{d}**	**30.4±0.17^{b}**	**29.33±0.07^{c}**	**29.25±0.40^{c}**	**28.41±0.10^{d}**
Unsaturated fats/ saturated fats*								
U/S	**3.56±0.07^{c}**	**4.05±0.03^{a}**	**3.85±0.00^{b}**	**3.70±0.03^{bc}**	**3.83±0.00^{b}**	**3.77±0.09^{b}**	**3.75±0.19^{b}**	**3.73±0.06^{b}**

*Values are mean ± standard deviation in triplicate. Samples with different letters are significantly different ($p < 0.05$).

460 *3.4. Phytic acid analysis (PA)*

461 Table 3 depicts a 17.43% reduction in PA due to the simple wash. Phytate content is mainly present
462 in the form of water-soluble salts such as sodium and potassium phytate and evidently it can be
463 reduced in RB through a phenomenon namely passive diffusion of water-soluble phytates during
464 exposure to water (Liu et al., 2019). This effect was reinforced by soaking after simple wash (as in
465 SWN) which reduced the PA content of the W by 26%. Generally, soaking can decrease the amount
466 of PA by two mechanisms whereby both account for our results. Firstly, by activation of phytase
467 enzyme which is responsible for hydrolysis of higher inositol phosphates to lower ones and even
468 myo-inositol and inorganic phosphates. Secondly, as mentioned above, by depletion of PA through
469 dissolution of water-soluble PA salts during soaking (Liu et al., 2019). Interestingly, acidic pH
470 reduced the PA even more than soaking at neutral condition indicating phytate phosphorus was highly
471 soluble at low pH. Comparatively, higher phytic acid content at basic condition relative to acidic
472 indicated lower solubility of PA at basic pH. Although, PA content was still lower than initial Washed
473 material (W). This phenomenon has been attributed to the presence of other salts, such as calcium
474 phytates and carbonates which have been entrapped by zinc or copper phytates inducing lower
475 solubility (Canan et al., 2011). Our pH results on PA solubility are confirmed by the study performed
476 by Grynspan and Cheryan (1983) who also concluded that increasing pH above 4 causes a drop in
477 PA solubility, whereas at pH above 7 the complexes will re-solubilize and the solubility again
478 increase. They explained that at high pH, there are enhancement on the amount of ionized hydrogen
479 on the low-calcium phytates molecules leading to their solubility and reduction elevation.

480 The ultrasound treatment significantly reduced the phytic acid content of the RB compared to their
481 not sonicated parallel sample. A 7, 11 and 23% decline of PA in ultrasonicated samples (UWA, UWN
482 and UWB) compared to just soaked samples (SWA, SWN and UWB) highlights ultrasound as a
483 driving force. It is apparent that acoustic effect of cavitation lead to disruption of surface material,
484 increasing the area and extraction rate of PA compounds into solvent. Interestingly, the two-way
485 ANOVA analysis of pH and ultrasonic treatment (Supplemental Table. 5) highlights the significant

interaction, or in other words, synergistic effect of the two treatments ($p < 0.05$). Sivakumar et al. (2004) also indicated that application of ultrasound in soaking process could decrease the time by up to 75% and in our case, the possibility of facilitating pH effect was confirmed by ultrasound.

Table 3
Effect of pH and ultrasound treatments on phytic acid concentration.

Sample Type	Phytic acid concentration (g/kg)
Control (C)	1.745±0.028[a]
Washed (W)	1.461±0.003[b]
Soaked Washed at Neutral pH (SWN)	1.059±0.028[c]
Ultra-sonicated Washed Neutral at pH (UWN)	0.986±0.037[d]
Soaked Washed at Acidic pH (SWA)	0.709±0.026[f]
Ultra-sonicated Washed at Acidic pH (UWA)	0.633±0.024[g]
Soaked Washed at Basic pH (SWB)	0.935±0.018[e]
(Ultra-sonicated Washed at Basic (UWB)	0.718±0.005[f]

*Values are mean ± standard deviation in triplicate. Samples with different letters are significantly different ($p < 0.05$).

3.5. Heavy metals analysis

Fig. 2 presents metals concentration in RB before and after all treatments. It should be noted that the Hg content in all samples were recoded zero and pointed to its lower concentration than LOQ; hence, not reported throughout this manuscript.

Among As, Cd and Pb, the highest decline by washing was observed in Pb (60%), followed by As (40%), Zn (32%) and Cd (27%) which was also reported by Sharafi et al.(2019). Liu, Zheng, and Chen (2018) also noted higher loss of Pb, Cd and As from the aleurone layer and outer layer of endosperm in rice which is similar to our polish fraction sample. Soaking W caused low but significant reduction in Pb (12.00%) in SWN, while no significant change was observed for As, Cd and Zn. This property is also reported by Sharafi et al. (2019) who indicated that soaking rice for a

duration of 1 h after washing was not adequate for removal of the toxic metals and longer time was suggested.

The effects of acidic, neutral and basic pH (2, 6 and 9) on the reduction of heavy metals were also investigated. Due to the differences in optimum pH for the solubility of metals, they showed inconsistent behavior towards various pH values. In terms of Pb, solubility increased with reducing pH as it was also shown by Deng et al (2009). Meanwhile, increased pH induced higher accumulation of Pb. Similar trend has been observed for Zn, whereby, the lowest accumulation was detected at acidic pH rather than basic which showed that Zn was highly dissolvable in acidic rather than neutral and alkaline condition. The possible reason is that lowest solubility for Pb and Zn would be achieved at pH 9 and 8.5, respectively (Ayres, Davis, & Gietka, 1994). Adding OH^- and increasing pH up to 9, as in alkali treatment, more Zn^{2+} or Pb^{2+} will react with hydroxide and form zinc hydroxide ($Zn(OH)_2$) or lead hydroxide ($Pb(OH)_2$) solids with lower solubility to Zn^{2+} and Pb^{2+} (Ayres et al., 1994).

On the contrary, alkaline pH was found to be more effective on the reduction of As and Cd rather than acidic pH. In case of As, this phenomenon has been attributed to the facilitated solvation of trioxide form of arsenic (As_2O_3) in alkaline environment to produce arsenite (AsO_2) (Hao, 2010). In terms of Cd, similarly, by increasing pH its removal was facilitated. Jha, Iyengar, and Rao (1988) also indicated that cadmium produces soluble complexes with hydroxide. Up to pH 7.5, cadmium exists in the form of Cd^{2+}, while by increasing pH within the range of 7.5-9, this divalent cation transforms to monovalent cation ($Cd(OH)^+$) with higher solubility attribute. By increasing pH to 9-11, the concentration of $Cd(OH)_2$ increases which is a more soluble form.

Ultrasound application alone (at neutral pH 6) and in combination with acidic and alkaline pH was able to reduce the heavy metal contents more efficiently than its parallel non-ultrasonicated sample. The exceptional has been observed for As at neutral and acidic pH which might be due to its very low solubility at those pH. Among all discussed heavy metals, ultrasound found to be more effective on the reduction of Pb at acidic solutions and less practical in reducing As at neutral and acidic solutions

(Fig. 2). The same results were also obtained by Porova et al. (2014) who reported the highest and lowest reduction percentages of Pb and As, respectively, in the milk treated by ultrasonic. Furthermore, two-way ANOVA analysis indicated a significant interaction between ultrasound and pH treatments for the heavy metals reduction ($p < 0.05$); while, mitigation in Cd was not synergistically affected ($p > 0.05$) (Supplemental Table. 5).

3.6. Coliforms quantitative analysis

Distribution of coliform counts in RB samples are provided in Table 4. Control rice bran (C) contained significantly more coliforms than all other samples with the exception for W and SWB. The 0.35 log CFU/g increase in W might be due to the leaching out of starch and soluble solids during washing process leading to a subtle increase in the ratio of bran to starch in cultured W. Hence, the elevation in W emphasizes that coliforms mainly accumulate in bran rather than starchy section of rice.

This effect of washing was more intensified around 62% reduction of coliforms by soaking at neutral pH as in SWN sample. A potential explanation could be longer exposure of RB surface to water; hence, water can penetrate completely into inner layers of RB, where coliforms highly accumulated. Ultrasonication of sample in similar condition as SWN resulted to diminish coliforms almost to the half in UWN. This signifies that ultrasonication at neutral pH was successfully able to inhibit coliforms growth.

Soaking Washed rice bran at Acidic pH (SWA) lowered the coliforms growth to < 1.0 log CFU/g. According to Kim and Ndegwa (2018) the optimum pH range for foodborne pathogens growth is between 7 and 9.Therefore, treatment at extreme acidic pH could impose inhibitory effect on coliforms growth and expectedly no colony could be detected for UWA at 10^{-1} dilution. Comparing to W, soaking at basic pH did not show a significant reduction in coliform counts of SWB and only by ultrasound treatment the same sample experienced decline in coliforms. Similarly, Kim and Ndegwa (2018) also observed the highest bacterial growth rate at pH 9. Nevertheless, efficiency of

ultrasonic on coliforms reduction is evident even at alkaline condition. It can be attributed to the bactericidal effects of ultrasound at frequency of 20 kHz because of its ability to lyse microbial cells as a result of cavitation. The inhibitory effect of ultrasound treatment on microorganisms such as coliforms is reviewed by Huang et al. (2017), who mentioned ultrasound disturbs cell walls and cytoplasmic membranes as well as bacterial capsules. Furthermore, ultrasound damages intracellular components including cytoplasmic shrinkage and disruption of DNA structure. It should be remarked that the two-way ANOVA analysis showed that reduction of coliforms was synergistically ($p < 0.05$) affected by the function of pH and ultrasonic treatments (Supplemental Table. 5).

Table 4
Effect of pH and ultrasound treatments on coliforms growth.

Sample Type	Coliforms* (log_{10} CFU/g)
Control (C)	5.30 ± 0.07^{b}
Washed (W)	5.65 ± 0.02^{a}
Soaked Washed at Neutral pH (SWN)	2.11 ± 0.03^{d}
Ultra-sonicated Washed Neutral at pH (UWN)	1 ± 0.00^{e}
Soaked Washed at Acidic pH (SWA)	ND
Ultra-sonicated Washed at Acidic pH (UWA)	ND
Soaked Washed at Basic pH (SWB)	5.62 ± 0.01^{a}
(Ultra-sonicated Washed at Basic (UWB)	2.43 ± 0.03^{c}

ND means not detected. (Coliform counts < 1.0 log CFU/g).
*Values are mean ± standard deviation in triplicate. Samples with different letters are significantly different ($p < 0.05$).

4. Conclusion

In this study, application of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran were investigated. To conclude, .water washing of rice bran demonstrated lower enzymes activity, FFA, PV, phytic acid and heavy metals contents but higher coliforms growth rate on rice bran. It was found that the combination of acidic pH and ultrasound was the most effective technique in order to reduce lipase and lipoxygenase activity. Although, ultrasound waves possess an adverse impact on peroxide value. Moreover, the

lowest concentration of antinutrient phytic acid and toxic metals was achieved by sonication at acidic pH. The inhibitory effect of pH and ultrasonic treatments showed no coliform growth at acidic pH with the presence or absence of ultrasound. Therefore, it may be suggested that optimization of combined ultrasound and pH process seems as a practical and non-thermal procedure in order to improve rice bran quality considering different aspects. Afterwards, the treated rice bran can be used as an ingredient for the production of functional foods and rice bran oil extraction. Further research on the process optimization using different varieties of rice bran and edible acids or evaluation of nutritional aspects on stabilized rice bran may be necessary to achieve to a value-added processing of rice bran with economical and energy saving benefits.

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Figure captions

Fig. 1. Schematic view of sample preparation and their analytical evaluation.

Fig. 2. Effect of pH and ultrasound treatments on the heavy metals concentration. (A) Zinc Concentration (µg/kg); (B) Arsenic Concentration (µg/kg); (C) Cadmium concentration (µg/kg); (D) Lead Concentration (µg/kg). Values are mean ± standard deviation in triplicate. Samples with different letters are significantly different ($p < 0.05$).

Control (C), Washed (W), Soaked Washed rice bran at Neutral pH (SWN), Ultrasonicated Washed rice bran at Neutral pH (UWN), Soaked Washed rice bran at Acidic pH (SWA), Ultrasonicated Washed rice bran at Acidic pH (UWA), Soaked Washed rice bran at Basic pH (SWB), Ultrasonicated Washed rice bran at Basic pH (UWB).Values are mean ± standard deviation in triplicate.