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Extraction and nutritional properties of protein derived from rice (*Oryza sativa*) based distillery byproducts:a potential substrate for food formulation

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ABSTRACT

Distillery byproducts, Distiller's Dried Grains with Solubles (DDGS) and Wet Solids (WS), are rich in fermented proteins thus, can be exploited to generate high value-added proteinaceous products. The present investigation aims to study the physical properties of DDGS and WS including Thermogravimetric Analysis (TGA) and X-Ray Diffraction (XRD). Protein fractionation and extraction from DDGS and WS have been carried out where, predominant fractions were glutelin and prolamin, having the yield of 81.75% and 75.02%, respectively. Furthermore, different protein extraction methods (solvent-based extraction, ultrasonication and enzyme-mediated) were compared for protein recovery where, aqueous-ethanol extraction resulted in highest yield of 47.7 and 59.8% w/w for DDGS and WS, respectively. In addition, determination of molecular weight of proteins and their purification were performed by SE-HPLC and SDS PAGE. This study is an attempt towards scientific explorations and innovative exploitations of distillery byproducts.

1. Introduction

The crop-based bioethanol production has increased tremendously over the year's despite of the controversy of food vs. fuel. It has been predicted to produce 42 million metric tons of DDGS (Distiller's Dried Grains with Solubles) as byproduct, by utilizing approximately 14% global cereal crops for ethanol production (OECD-FAO Agricultural, 2012). Therefore, the production of DDGS is massive and is challenging mission for distillery industries to transform/bioprocess it into high value products; otherwise its prolonged storage and disposal into environment could cause severe ecological issues like eutrophication in water bodies and soil infertility (Singh et al., 2018a, b).

DDGS is highly rich in fermented proteins (26.0–31.7%) in comparison to lipid (10.2–11.4%) and fibre (9.6–10.6%) (Liu, 2011). With the rising concern of consumers and advancement in biotechnology, fermented proteinaceous products are in high demand as they increase bioavailability/bioabsorbability of peptide and amino acids in human cells (Singh et al., 2018a, b). Moreover, it could open several opportunities to exploit these extracted proteins in various ways such as in formation of protein film, protein powder, protein isolates, protein concentrate etc. For human consumption. However, rice-derived DDGS and WS remain unexplored for protein availability and its nutritious characteristics. Hence, successful exploitation of rice based distillery byproducts (DDGS and WS) using environmentally benign processes is important for valorization that has a good impact on market value.

In this study, fractionation of rice DDGS and WS (albumin, prolamin, globulin and glutelin) was performed to find out the major protein fraction available in the distillery byproducts. Water insoluble proteins *viz.* Glutelin and prolamin were primarily targeted in the extraction process. Further, five different methods viz. Aqueous-ethanol, alkaline-aqueous ethanol, pH-shifting, ultrasonication treatment and alkaline-protease were compared for the extraction of as-mentioned protein fractions. A 'one-variable-at-a-time' approach has been used to optimize aqueous-ethanol and alkaline-aqueous ethanol extraction processes for the parameters like temperature (°C), concentrations of solvent (%) and sodium metabisulphite (%) (SMB). SE-HPLC was conducted to purify the obtained protein fractions whereas, SDS-PAGE was performed to determine molecular weight (MW) of all the protein fractions. Amino acid analysis of extracted DDGS protein isolates (DDGSPI) and glutelin was also carried out. This study is an attempt to harness the potential properties available within the byproducts derived from rice-based distillery for formation of value-added protein products.

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2. Methods

2.1. Raw materials

Rice DDGS and WS were generously provided by IFB-Agro, a rice-based distillery plant in West Bengal, India. The samples were stored at 4 °C after procurement.

2.2. Characterization of rice DDGS and WS

2.2.1. Biochemical characterization

The particle size of DDGS was reduced with mortar and pestle, thereby passing it to 0.5 mm sieve to obtain uniform size of the sample. The standard AOAC (Association of Analytical Communities) method was used to estimate moisture and ash content (AOAC, 2000); whereas, total protein was estimated by Kjeldahl analysis (using N × 6.25 conversion factor) (AOAC, 2000) and fibre content was analysed by the protocol of Prosky et al., (1988). Anthrone method was used to estimate total carbohydrate and starch (Ludwig and Goldberg, 1956). For lipid estimation, hexane-mediated Soxhlet apparatus was used. Water activity (aw) and pH activity for DDGS and WS samples were detected by AquaLabs 4TE and EuTech Instrument pH tutor, respectively.

2.2.2. Elemental composition of DDGS and WS

The CHNS determination of rice DDGS and WS was done using M/s Elementar, VarioMicrocube, Germany. The samples were dried in the oven at 105 °C for 4–5 h till constant weight has been attained. Thereafter, samples were sealed in capsule and combusted in a reactor at a constant flow of carrier gas (helium). Oxygen was calculated by subtracting the sum of all elements from 100.

2.2.3. Physical characterization

2.2.3.1. Thermogravimetric analysis (TGA). Thermal analysis of rice DDGS was measured by Thermogravimetric Analyzer (TA-Q600SDT). TGA was conducted according to ASTM method E1641-07 in presence of nitrogen flow (20 mL per min) with a heating rate of 10 °C per min ranging from 30 to 800 °C (ASTM, 2007).

2.2.3.2. X-ray diffraction (XRD). XRD was conducted for the rice DDGS to study crystallinity index using a XRD1710 equipment with Cu Kα radiation where α = 1.79Å, at 40 kV and 20 mA. Diffractogram was scanned at a diffraction angle (2θ) from 0 to 90° with a scanning speed of 3° per min (Dai and Fan, 2010).

2.3. Protein fractionation by osborne method

A modified Osborne fractionation procedure was carried out with the aim of extracting rice proteins from DDGS and WS (Osborne, 1907). For all the extractions, 1 g of sample was dissolved in 10 mL of solvent and stirred at 300 rpm for 30 min. Collected aliquots were centrifuged at 3500 rpm for 20 min. The supernatants were pooled in an eppendorf labelled as albumin, globulin, prolamin and glutelin, for analyzing protein content and extraction yield. Bradford assay was used to estimate protein content (Bradford, 1976). Further, supernatants were lyophilized and kept at −20 °C for SE-HPLC and molecular weight determination was carried out using SDS-PAGE.

2.4. Extraction of protein

2.4.1. Extraction with aqueous-ethanol

From the previously stored raw samples, 10 g of powdered sample (section 2.2.1) was solubilised in hexane (100 mL) at 37 °C for 4–5 h to remove lipid content. Defatted samples were solubilised in 100 mL of aqueous-ethanol (20–90%,w/v) and incubated under continuous shaking (500 rpm) followed by centrifugation (8000 rpm for 15 min). Thereafter, reducing agent (SMB, 0.25–2.0%) was added in aqueous-ethanol followed by repeated centrifugation for determination of extractability of protein. Similarly, temperature variations (50–100 °C) was carried out to determine the efficiency of protein extraction. The extracted proteins were estimated by Bradford method (Bradford, 1976).

2.4.2. Extraction with alkaline-aqueous ethanol

Protein extraction was carried out by varying ethanol concentration (20–90%,v/v) in 1N sodium hydroxide followed by protein estimation (Bradford, 1976). The other parameters mentioned in section 2.4.1 were same for alkaline-aqueous ethanol extraction process.

2.4.3. Extraction with the effect of pH shifting

DDGS and WS were mixed with sodium phosphate buffer (10mM, pH 7.0) separately in 1:10 (w/v) ratio. The pH of the solution was adjusted from acidic to alkaline pH ranging 8–12.5, at an interval of 0.5 with 0.1N NaOH. The solutions were kept in a shaker at 300 rpm for 30 min at 25 °C. Centrifugation was performed at 3000 rpm for 15 min, and supernatants were pooled for protein estimation.

2.4.4. Extraction using ultrasonication

Ultrasonication treatment to DDGS and WS was performed by sonicator (Labman PRO 650). A 5 g of sample (DDGS and WS separately) was dissolved with 50 mL of sodium phosphate buffer (pH 7) in a 150 mL beaker and sonotrode was inserted into it. The treatment conditions were: power output-750 W, frequency-25 kHz, 25 °C by varying the time period: 10–60 min (pulse durations of on-time 9 s and off-time 1 s). Ultrasonic treated samples were centrifuged and supernatant were collected for estimation of protein (Bradford, 1976).

2.4.5. Impact of protease on protein extraction

Extraction of protein from the powdered DDGS and WS (section 2.2.1) were carried out using protease isolated from *Aspergillus awamori Nakazawa* MTCC 6652 (Negi and Banerjee, 2011). One gram of DDGS and WS were taken separately and mixed with 10 mL protease (513 U/mL), stirred at 520 rpm for 30 min, pH 8 at temperature ranging from 25 to 60 °C followed by centrifugation (5000 rpm for 10 min). The supernatant collected was subjected for protein estimation (Bradford, 1976).

2.5. Fourier transform infrared spectroscopy (FTIR)

To get information about secondary structure of extracted proteins i.e. DDGSPI and glutelin were scanned in the range of 400–4000 cm-1 with a Nicolet Nexus 670 FTIR spectrometer (Thermo Electron Corp., Madison, WI). The samples were ground thoroughly with KBr in a smooth mortar.

2.6. Size exclusion-high pressure liquid chromatography (SE-HPLC)

SE-HPLC of the extracted samples (albumin, globulin, prolamin, glutelin and DDGSPI) was performed (Agilent technologies 1100 series, GF-250 Agilent Zorbax column (4 μm, 9.4 × 25mm)) according to the protocol of Sviridov et al. (2006) with a slight modification; where gradient elution was adopted with binary mobile phase of 0.1 M phosphate buffer (pH 7.4; A) and 0.1 M NaCl (B) at a flow rate of 0.5 mL/min using UV–Vis detector (290 nm).

2.7. Sodium dodecyl sulphate polyacrlyamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by following the protocol of Laemmli (1970) to determine the exact molecular weight of DDGS protein fractions (albumin, prolamin, globulin and glutelin) and DDGSPI. The

Table 1
Composition characterization and fractionation of DDGS and WS.

(%, db)	DDGS	WS
Dry matter	90.3±(0.8)	30.5±(0.9)
Ash	5.0±(0.7)	3.4±(0.4)
Crude Protein	32.7±(1.2)	29.8±(1.1)
Total Carbohydrate	35.1±(0.6)	44.3±(0.1)
Total Starch	3.6±(0.1)	3.1±(0.7)
Total fibre	19.50±(1.3)	16.4±(1.0)
Crude Lipid	4.1±(0.5)	3.0±(0.2)
pH	4.5–5.0	3.2–4.0
Water activity (a_w)	0.45 ± 0.1	0.97 ± 0.3

Values in bracket represents± SD, n = 3; db: dry weight basis.

standard protein marker (10–200 kDa) (Sigma), was used for molecular weight estimation of the unknown proteins (Matsumoto et al., 2019).

2.8. Amino acid profiling of DDGSPI and glutelin

The nutritional quality of any protein is predominantly governed by its amino acid composition. The amino acid composition of DDGSPI and glutelin was analysed by using a Model L-8900 Amino Acid Auto-Analyzer (L-8900, HITACHI, Japan).

2.9. Statistical analysis

All experiments were carried out in triplicates and the data was reported as means of three values. Design-Expert (Version 8.0.6, Stat-Ease) and Origin Pro (version 8.0) software (Stat-Ease, Inc., Minneapolis, USA) were used for analyzing results. Significant differences were defined at the $P < 0.05$ level between the samples.

3. Results and discussion

3.1. Compositional analysis

To evaluate the rice based DDGS and WS composition, a series of experiments have been conducted (Table 1). The ash and total solid content were found to be higher in DDGS as compared to WS. It may be due to the high availability of inorganic matter present in DDGS. While estimating the crude protein, it has been seen that 32.7% (w/w) protein was obtained in raw DDGS, whereas, 29.8% (w/w) protein was recorded in case of WS. The high availability of protein component is due to fermentation with yeast. On the other hand, protein content in wheat and corn DDGS was lower than in rice DDGS (Cookman and Glatz, 2009; Cozannet et al., 2010). This variation may be due to the difference in nutritional composition of grains varieties.

Rice being staple starchy cereal made it obvious to estimate the leftover starch content in DDGS and WS. The total carbohydrate content of DDGS and WS were found to be 35.1% (w/w) and 44.3% (w/w), respectively. It was expected that a lower % of starch will be present in DDGS and WS because of conversion of glucose to ethanol through anaerobic fermentation. To quantify the concentrations of residual starch in DDGS and WS, experiments have been carried out. As expected, residual starch content was less (3.1–3.6%, dry weight basis) in both the samples. This is due to maximum utilization of starch during ethanol production. The lipid content of DDGS and WS is less (3.0–4.1%, on dry weight basis) which indicates that it has good solubility in polar solvents. While further processing of the DDGS and WS, it was felt that water activity is an important parameter to be studied. From Table 1, it can be revealed that the water activity (aw) of DDGS (0.45) is less than WS (0.97). From this result, it can be inferred that DDGS is safe from microbial attack as no microbes can grow in food sample with water activity less than 0.6 (Tapia et al., 2007). In contrast, WS has high water activity (high moisture content, Table 1) which is prone to high microbial attack.

Table 2
Elemental analysis of DDGS and WS.

(%)	DDGS	WS
C	46.12	43.63
H	6.01	6.75
N	6.91	6.66
S	0.39	0.31
O	40.55	42.65

C-Carbon, H-Hydrogen, N-Nitrogen, S-Sulphur, O-Oxygen.

3.2. Elemental composition of DDGS and WS

Table 2 shows the elemental analysis of DDGS and WS. The findings of the present study have been validated with those of Morup et al. (2012), where corn and wheat DDGS were utilized. As it is a plant-based substrate, the predominance of carbon percentage was obvious and it forms higher degree of cross-linking with hydrogen. It also illustrates the possibilities of utilizing DDGS and WS in different food formulations, as it contains substantial amount of elements which can bind with functional groups of other food ingredients to form a uniform shape. Flodman (2013) has utilized corn DDGS for the production of cellulose due to its high % of carbon. The possible reason for detection of low sulphur percentage may be because of less exposure of thiol (-SH) containing amino acids which are embedded inside the hydrophobic core of protein.

3.3. Physical characterization

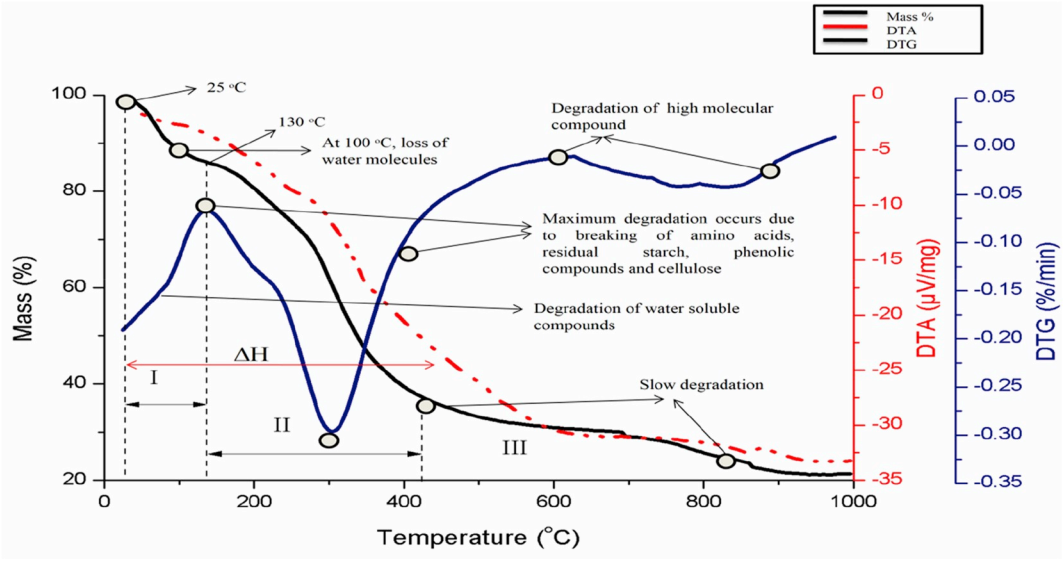
3.3.1. Thermal analysis

Thermogravimetric analysis (TGA) was carried out to evaluate the ongoing conformational changes of macromolecules, transition stages of macromolecules during the process in terms of enthalpy, temperature, and heat capacity. Fig. 1a shows TGA mass loss, DTA and DTG curve as plotted against the temperature. In the first stage of decomposition, loss in mass percentage starts from around 25 °C and this phase is considered as drying phase. As depicted from Fig. 1a, there is a sudden decline in mass percentage at 100 °C due to loss of water molecules. TGA analysis gives a wide temperature range between which maximum thermal degradation of the sample is taking place, i.e. from 130 to 420 °C. The transition in temperature was observed due to the breakage of covalent bonds present in amino acids as well as breaking of residual starch, cellulose, and phenolic acid. Cleavage of disulphide (O–N and O–O) bonds present in protein moieties also occurs in this temperature range. Degradation of DDGS above 420 °C shows slower mass loss, probably due to the thermal degradation of high-molecular weight components like lignin. Studies on maize has shown that during thermal analysis, the loss of moisture takes place between 60 and 110 °C, representing a mass loss of about 10%.

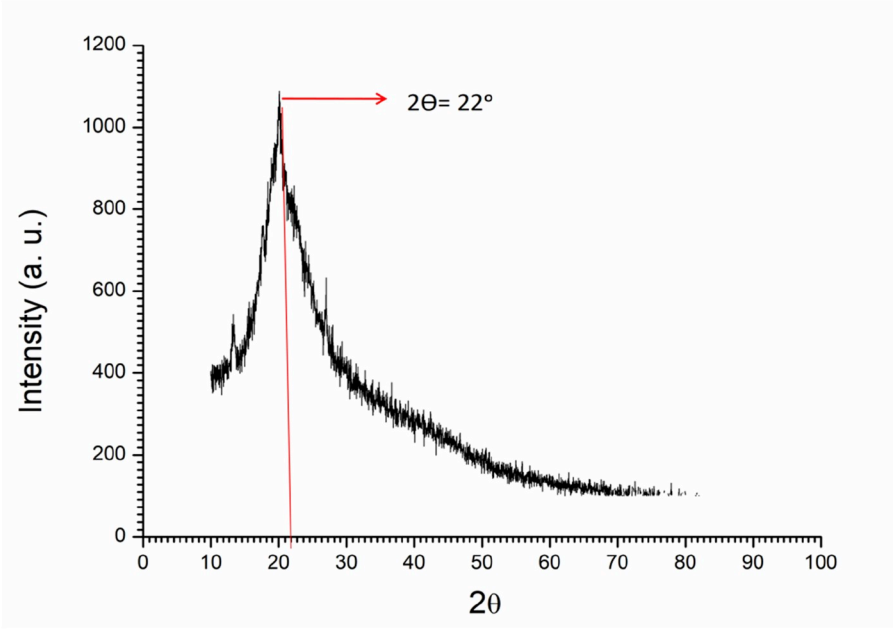
In DTG analysis (Fig. 1a), the major peak was observed at 300 °C during pyrolysis (130–420 °C) where degradation of amino acid, cellulose, residual starch, phenolic compounds, and hemicellulose took place (Zarrinbakhsh et al., 2013). The shoulder peaks to the left of the amino acid belongs to the breaking of water soluble component viz. Glucose and xylose, organic acids (succinic and lactic acid), and fermented byproducts such as glycerol and butanediol. Volatile components and other water soluble biomolecules lie in the range of 100 to 200°C. To the right of the pyrolysis phase, degradation of insoluble proteins takes place (Zarrinbakhsh et al., 2013). Thus, knowledge about thermal analysis of any substrate is of vital importance as it plays a significant role in food industries, as most of the steps involved during food/feed formulation have heating processes that can change the nutritional and functional characteristics of the end products.

3.3.2. X-ray diffraction

The diffraction pattern was studied to recognize structural



(a)



(b)

Fig. 1. Physical characterization of rice DDGS by (a) Thermogravimetric analysis, (b) X-ray diffraction.

alterations in the amorphous and crystalline regions. For DDGS, a sharp peak was obtained at $2\theta = 22^{\circ}$ which shows that DDGS had 32.64% crystallinity, and rest of the sample is amorphous in nature (Fig. 1b). The high amorphicity indicates that the rice DDGS can be easily formulated with minerals, vitamins and other biomolecules that can serve as enriched food supplements. This is in accordance with the results reported by Wu and Munkvold (2008), where $2\theta = 22^{\circ}$ was observed using corn DDGS as a substrate.

3.4. Osborne fractionation of rice DDGS and WS

DDGS and WS samples were dissolved in different solvents (water, 0.5 N NaCl, 70% ethanol, 45% 1-propanol) to identify the solubility and nature of the protein present in DDGS and WS. From Table 3 it can be inferred that water soluble albumin fraction was obtained from total extracted protein that accounts for 10.09% (w/w) and 9.03% (w/w) in DDGS and WS, respectively. This suggests that only a small fraction of the protein is water soluble; whereas, rest of the major protein fraction

Table 3
Estimation of protein fractions available in rice DDGS and WS by modified Osborne method.

Estimation of protein fractions available in rice DDGS and WS by modified Osborne method		
Types of fractions	DDGS (%,w/v)	WS (%,w/v)
Water soluble (Albumin)	9.30	10.09
Salt soluble (Globulin)	3.61	3.23
70%ethanol soluble (Prolamin)	22.0	19.4
45% 1-propanol soluble (Glutelin)	40.11	49.03

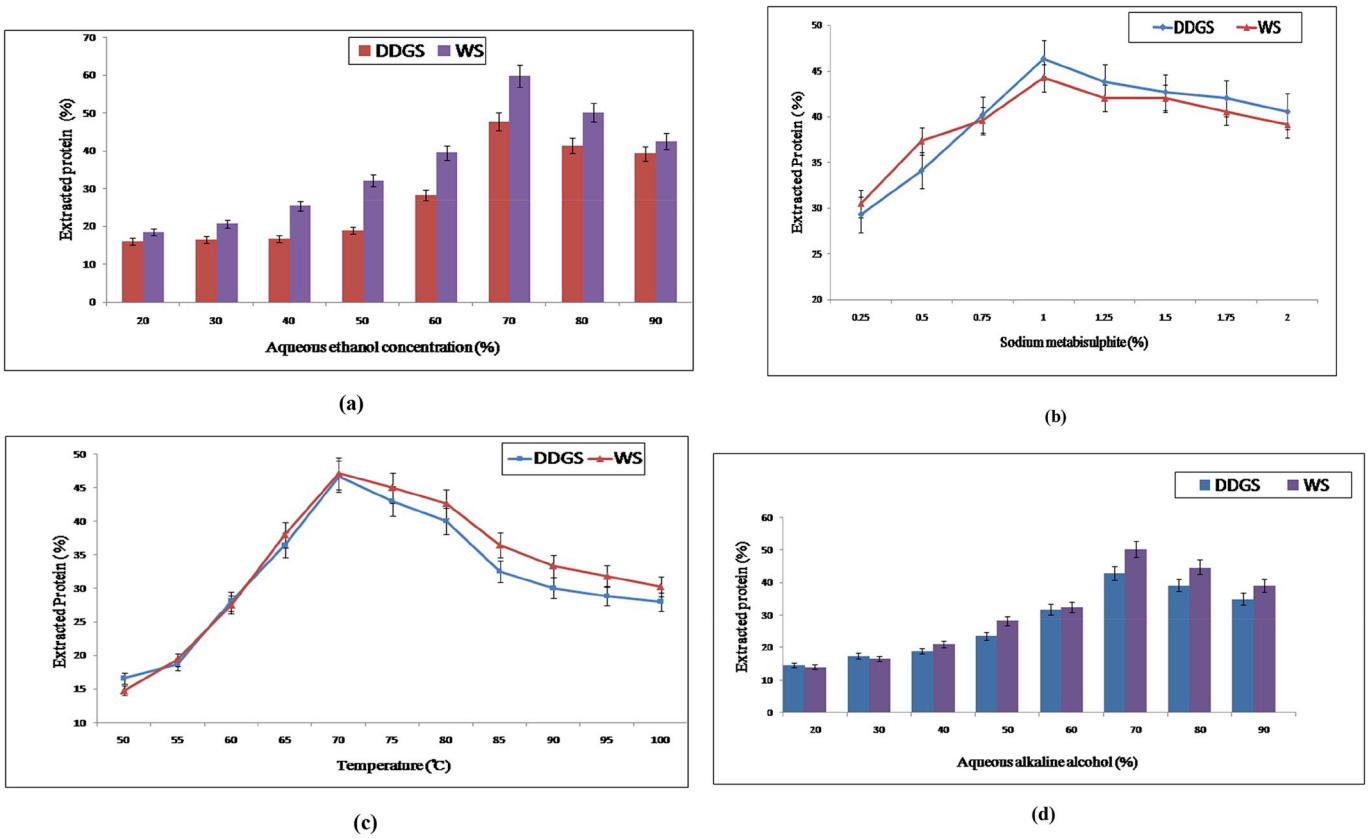


Fig. 2. Extraction of protein from rice DDGS and WS by 3 methods *viz.* Aqueous ethanol: (a) Effect of solvent concentration, (b) Effect of temperature (°C), (c) Effect of SMB %; Alkaline-aqueous ethanol: (d) Effect of solvent concentration, (e) Effect of temperature (°C); (f) Effect of SMB %; pH shifting (g); (h) Extraction with ultrasonication; (i) Enzyme-mediated extraction.

remained insoluble. The highest fraction of alkali-soluble glutelin was observed in DDGS and WS, which accounts for 49.03% (w/w) and 40.11% (w/w) respectively of the total extracted protein. Glutelins are the major storage protein available in the endosperm of rice and has highest fraction among all the available proteins. The solubility of glutelin is very poor because it aggregates profusely and joins with a disulfide bond, making it difficult to solubilize in any solvent other than alkaline solution. Alcohol soluble prolamin was found to be the second highest protein fraction available in all the extracted samples, which is in agreement with the findings of Muench et al. (1999). As illustrated in Table 3, prolamin fractions were found to be 19.4% (w/w) and 22.0% (w/w) in DDGS and WS, respectively. Similarly, globulins derived from salt solution (0.5N NaCl) have concentrations of 3.23% (w/w) and 3.61% (w/w) in DDGS and WS, respectively. The obtained yield for DDGS and WS were 81.75% and 75.02%, respectively. It can be inferred from the obtained results that modified Osborne fractionation procedure could be a feasible option for extracting proteins from the rice DDGS and WS.

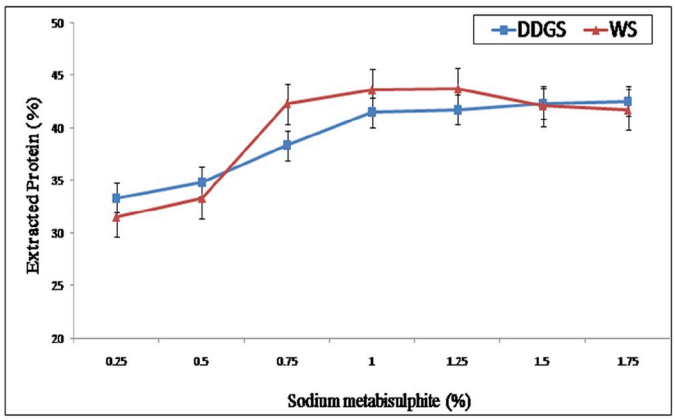
3.5. Protein extraction from rice DDGS and WS

For the efficient protein extraction with minimal loss, a selection of appropriate extraction technique is an important parameter. Based on the nature of the protein and its solubility, solvent-based extraction of the protein fractions using aqueous-ethanol, alkaline-aqueous ethanol, and pH-shifting methods were carried out effectively. Further, ultrasonication treatment and enzyme-mediated extraction were also conducted to find out the extraction efficiency. The obtained results were discussed in the following sub-sections.

3.5.1. Extraction by aqueous-ethanol

Initially, the experiment was designed by varying concentration of solvents from 20 to 90% (v/v). From Fig. 2a it can be observed that the maximum extraction of water insoluble proteins is at 70% (v/v) aqueous-alcohol. At lower concentration i.e. below 70% (v/v), the percentage of extracted protein was found to be low due to the lesser protein solubility. This might be due to the folded structure of protein molecules where peptide groups were buried inside and non-polar side chains were exposed on the surface. Similarly, the solubility of the protein decreases above 70% (v/v). The possible reason behind this observation could be denaturation of protein molecules occurred at higher concentration of organic solvent and also might be due to the change in dielectric constant of the medium. The decrease in dielectric constant increases the coulombic forces between the unlike charges of protein molecules and thus, lowering the solubility. Therefore, it can be inferred that 70% (v/v) aqueous-alcohol could be considered as the most suitable concentration for extraction of proteins from distillery byproducts. After fixing a solvent concentration, experiments were carried forward to determine the effects of SMB concentration and temperature on protein extraction. The percentage of SMB was varied in the range of 0.25–2.0%. From Fig. 2b, it can be observed that 1% SMB was found to be optimal for both the samples.

After fixing two parameters (solvent concentration and SMB), variation of temperature (50–100°C) was considered to find out an appropriate extraction condition. The extraction procedure carried out at 50°C showed low protein percentage. The extraction carried out at 70°C resulted in improved protein content of the samples, viz. 47.7% (w/w) in case of DDGS and 59.8% (w/w) in case of WS (Fig. 2c). A 1.46 fold increase in protein % was recorded in case of DDGS whereas, 2.0 fold has been increased in case of WS. Extraction at 90°C showed decreased



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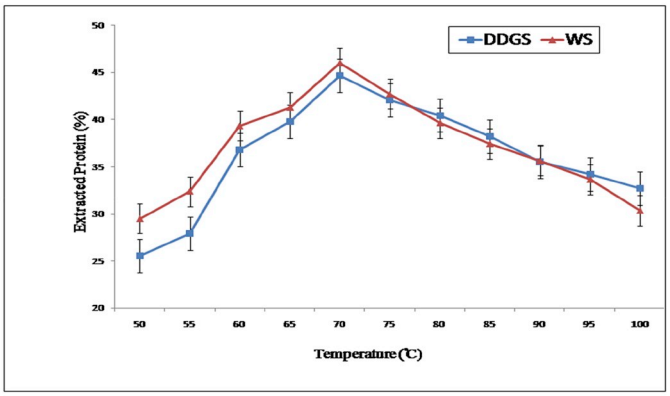

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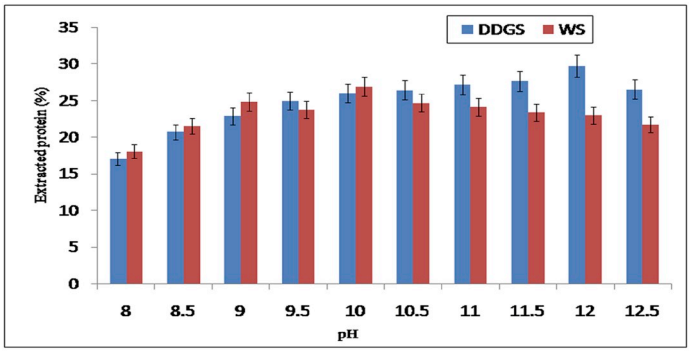

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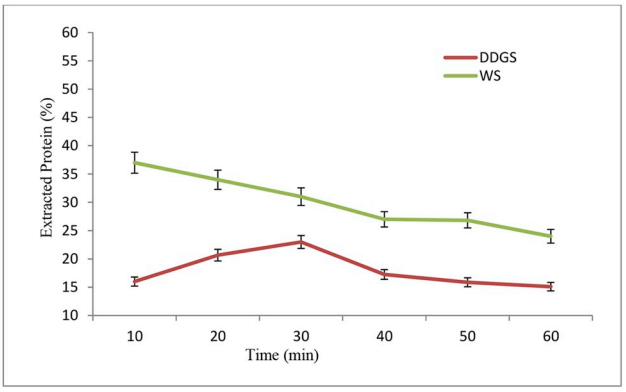

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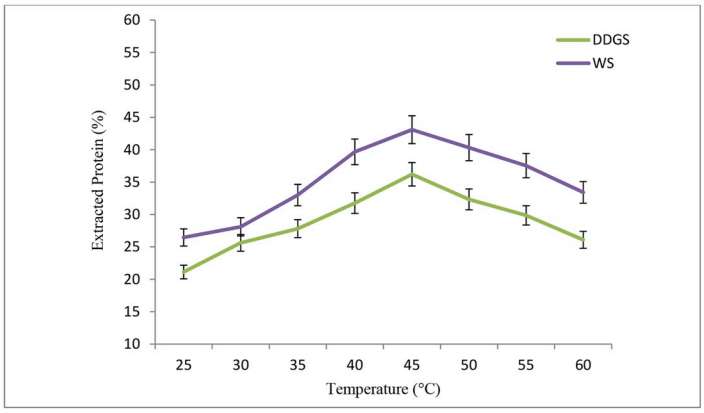

(i)

Fig. 2. (*continued*).

protein content compared to the extraction carried out at 70°C. In the presence of aqueous-ethanol solvent, the hydrophobic fraction of rice protein gets solubilised resulting in the disruption of low energy hydrogen bonds in the decreased dielectric constant of the medium. The decrease of the disulphide bonds is responsible for the solubilisation of smaller subunits present in prolamin and several low molecular weight glutelin subunits, which in turn makes the available proteins soluble in warm alcohol solution. Moreover, above 70°C, the disulphide bond rearrangement takes place. This is facilitated by a temperature-dependent unfolding of the protein's tertiary structure, ultimately reducing sample solubility in the extraction solvent. Though, the availability of the reports on the extraction of proteins from rice DDGS is negligible hence, the comparison is made with DDGS from other cereals. Anderson et al. (2012) studied an extraction process using 70% aqueous 2-propanol and aqueous ethanol in the mixture and extracted α-zein from maize DDGS. Similarly, Xu et al. (2007) established their work by taking corn DDGS as a primary substrate by using 70% ethanol with 0.25% sodium sulphite at pH > 5.

3.5.2. *Extraction by alkaline-aqueous ethanol*

A concentration of alkaline-aqueous ethanol has been varied from 20 to 90% (v/v). Generally, alkali (NaOH) and acid (HCl) were used to hydrolyze the complex protein into smaller peptide fragments, thus resulting in increased extractability and solubility. The maximum

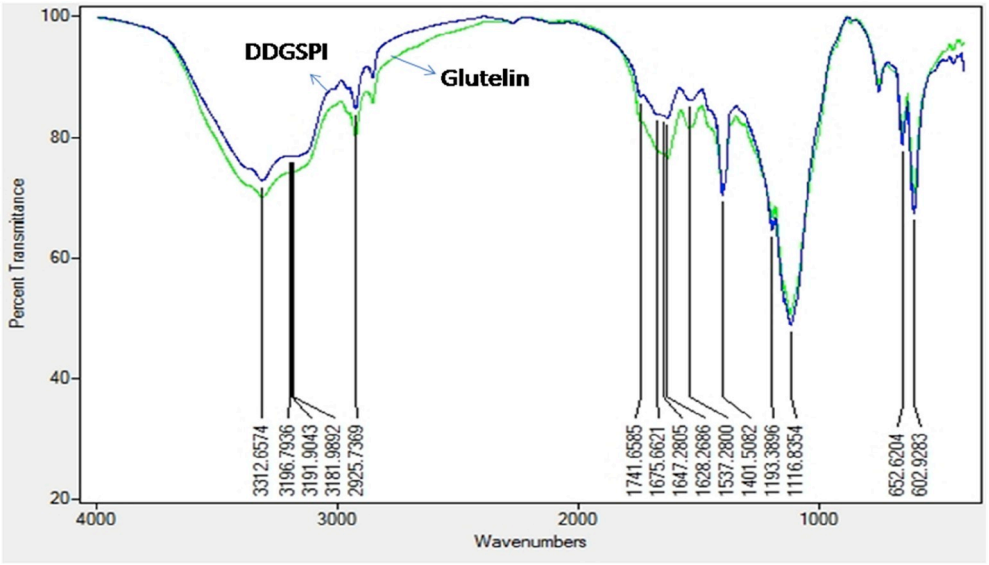

Fig. 3. FTIR spectrum of DDGSPI and glutelin.

extractability of protein was increased significantly ($P < 0.05$) at 70% (v/v) alkaline-aqueous ethanol (Fig. 2d). Similarly, SMB % was varied at different concentrations (0.25–2.0%, w/v). The maximum extraction was observed in the presence of 1.25% (w/v) SMB for both the cases (Fig. 2e). At 70°C maximum protein extractability has been observed for both the samples. Thereafter, with elevated temperature protein molecules were observed to be coagulated resulting in denaturation of proteins (Fig. 2f). The estimated protein contents were found to be 42.9% (w/w) and 50.3% (w/w), showing an increase in 1.31 and 1.68 folds for DDGS and WS, respectively.

3.5.3. Extraction by pH-shifting

Extraction in acidic pH did not show good extractability but upon varying the pH range from 8 to 12.5, the solubility of protein increased significantly under strong alkaline environment. The maximum solubility of DDGS (29.78 %w/v) and WS (26.96 %w/v) were observed at pH 12 and pH 10, respectively (Fig. 2g). Beyond pH 12, no further increase in protein % was observed in both the cases which may be due to the protein denaturation. Similar results were also reported by Bandara et al., (2011), who extracted triticale protein in the range of pH 8.5–12.0. Thus, from the above study, it can be interpreted that high alkalinity is required for better extraction of protein. WS gives high protein % at pH 10 because the substrate is in form of wet cake and it is not passed through steam dryer. Whereas, DDGS is dried at higher temperature which might lead to change in conformation of protein molecules, thus higher pH is required to increase the solubility of protein by resulting good extractability. The increase in protein extraction fold was 0.91 and 0.90 for DDGS and WS, respectively.

3.5.4. Ultrasonication

From Fig. 2h, it can be inferred that maximum protein extractability (23%,w/w) for DDGS was observed at 30 min beyond which there was a decreased trend. This might be due to the exposure of substrate to the ultrasonic environment for a longer time resulting in temperature rise in the solution causing significant degradation of proteins. Gu and co-workers conducted ultrasound-assisted fractionation of distillers' grain and reported similar results for protein degradation due to treatment for prolonged time period (Gu et al., 2019). For WS, maximum extractability was observed at 10 min (37%, w/w). WS, slurry leftover after distillation process, has small particle size and thus, less time required to breakdown. On the other hand, DDGS undergoes high temperature heating during its production that has resulted into change in conformational structure resulting denaturation of protein.

3.5.5. Enzymatic extraction

Enzymatic extraction (Fig. 2i) refers to extraction of protein from DDGS and WS. From the graph, it can be inferred that maximum extracted protein % was observed at 45 °C where, protein recovery from WS (43.09%,w/w) is more in comparison to DDGS (36.21%, w/w). The probable reason might be due to the presence of more denatured protein in DDGS which has resulted into less recovery. Tang et al. (2002) used protease and amylase in concoction and achieved 61.8% of protein from rice bran. While, Cookman and Glatz (2009) reported a reduction in protein content from 36 to 10% in corn DDGS after 2 h of digestion with Protex 6L. Protease can be an important extraction means for commercial exploitation provided its sustainability, availability and cost can be worked out.

From the above observations, it can be inferred that a suitable extraction conditions like temperature, solid/solvent concentration and reducing agent play a significant role for maximum recovery of protein from DDGS and WS. A lower protein recovery was observed in case of pH shifting as compared to the extraction with aqueous ethanol and alkaline-aqueous ethanol. Similarly, ultrasonication and enzyme-mediated extraction have resulted into less yield compared to the aqueous ethanol and alkaline-aqueous ethanol techniques.

Though the extracted protein % from WS was more compared to that of DDGS, yet it cannot be overlooked that WS contained more moisture than DDGS; resulting in the reduction of shelf-life (more water holding capacity, Table 1) and more prone to microbial growth. Therefore, transportation of WS could be a serious problem and thus DDGS may be considered as one of the potential substrates over WS for its good longevity. Moreover, DDGS might have a better application for producing high value-added products related to food formulations. Thus, to study the application of DDGSPI and glutelin (the major protein fraction obtained from the Osborne fractionation method) in food formulation, their secondary structure and nutritional quality was also estimated.

3.6. FTIR spectrophotometry

From the FTIR graph (Fig. 3), the stretches of the major functional groups and secondary structure of DDGSPI and glutelin has been analysed. Four major characteristic peaks were observed from FTIR analysis i.e. first at 3312.65 cm^{-1} corresponding to N–H primary stretching caused by flexural vibration frequencies of the intra- and inter-

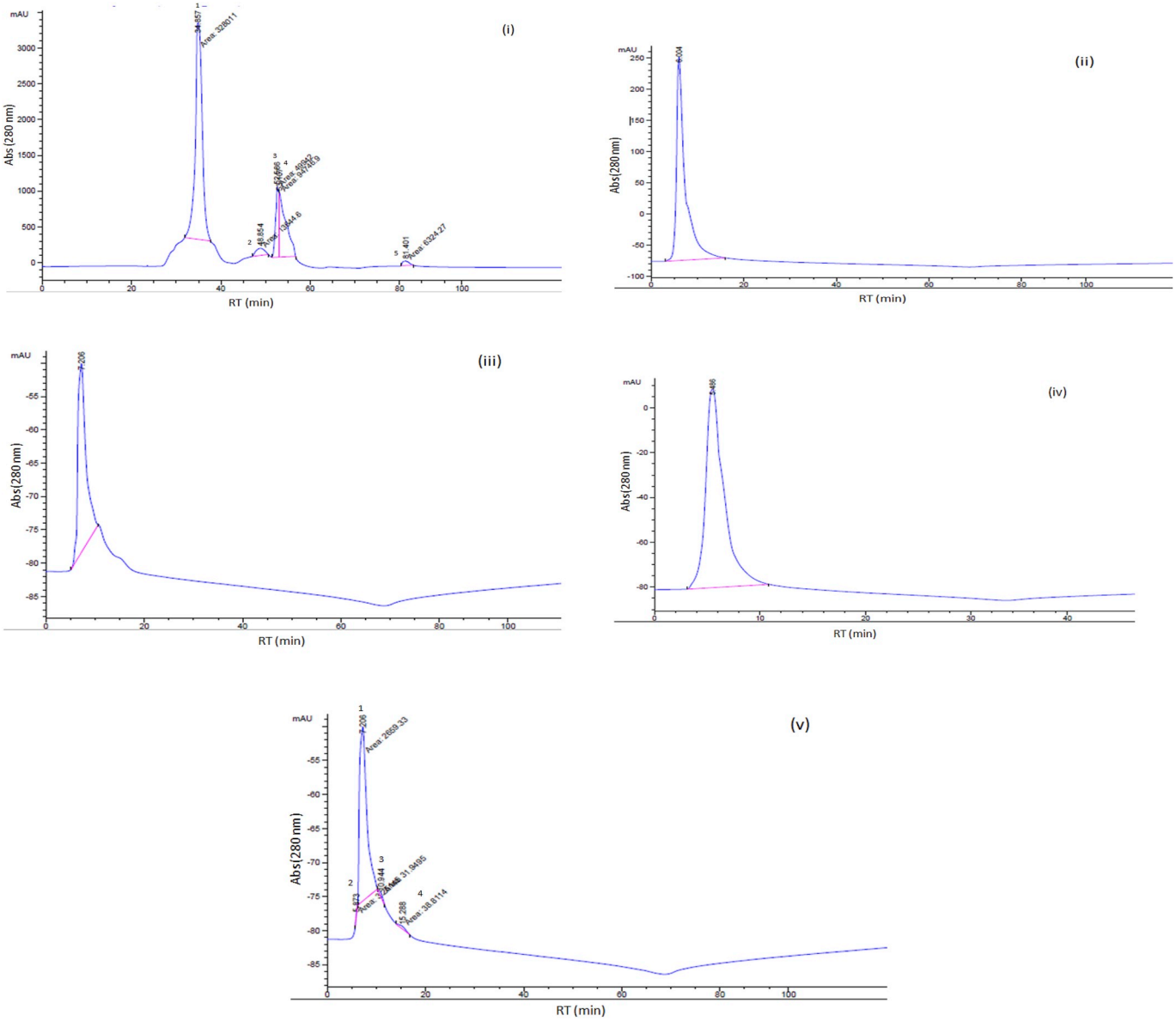

Fig. 4. SE-HPLC of DDGS sample and extracted DDGSPI (i) Albumin fraction; (ii) Globulin fraction; (iii) Glutelin fraction; (iv) Prolamin fraction; (v) DDGSPI.

molecular hydrogen bonds. The other peak at 2925.73 cm^{-1} corresponding to C–H vibrational stretching. The characteristic peaks of both DDGSPI and glutelin represent the Amide I and Amide II bands observed at 1647.28 cm^{-1} and 1537.28 cm^{-1}, respectively. The absorption associated with the Amide I band leads to stretching vibrations of the covalent bond (C═O) of the amide; Amide II band leads to bending vibrations of the N–H bond. Amide I and amide II peaks in the region between 1400 and 1700 cm^{-1} indicate the predominance of secondary structures α and β.

3.7. SE-HPLC of DDGS and DDGSPI

The chromatogram obtained from the SE-HPLC profiles of albumin, globulin, glutelin, prolamin and extracted protein isolates (DDGSPI) has been shown in Fig. 4 (i, ii, iii, iv and v). The albumin fraction was found to consist of five peaks where 3 major peaks i.e 1, 3 and 4 are having retention time of 34.85, 52.58, 53.10, and 2 minor peaks i.e 2 and 5 with retention time of 48.85 and 81.40 min. Whereas, globulin, glutelin and prolamin had single peaks each at 6.4 min, 7.2 min and 5.4 min, respectively (Fig. 4 ii, iii and iv). Protein isolates of DDGS was further analysed and the resultant chromatogram had one major (7.2 min) and three minor peaks (5.8 min, 10.9 min and 15.2 min). Based on the HPLC study, it can be inferred that the protein isolate extracted from DDGS has glutelin component. So, it can be concluded from the SE-HPLC profile that major fraction of water insoluble DDGS protein i.e glutelin has been isolated successfully.

3.8. SDS- PAGE

The detailed profiling of rice based DDGS and extracted protein isolates was carried out through SDS PAGE along with molecular weight determination by following the protocol of Matsumoto et al., (2019). Lane 1, 2, 3 and 4 represents albumin, globulin, prolamin and glutelin, respectively. The protein standard was loaded in the lane 5 ranging from 10 kDa to 200 kDa and in lane 6 extracts of DDGSPI was loaded (Fig. 5). It can be observed from the gel that the separation of protein bands is varying from 10.11 to 85 kDa. While analyzing lane 1, it has been observed that two bands having MW of 12.6 and 10.11 kDa corroborates the presence of albumin in DDGS. Similarly, lane 2 demonstrated two bands having molecular weight of 35.53 and 10.11 kDa indicating the

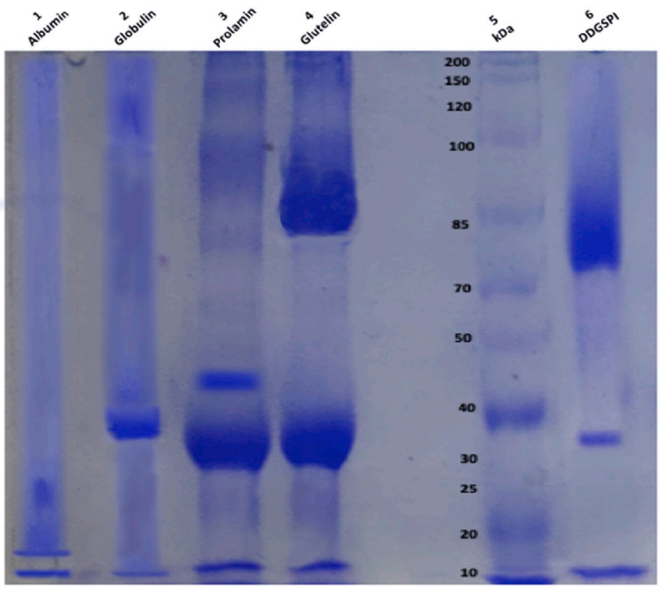

Fig. 5. SDS-PAGE analysis of DDGS protein fractions from Osborne method and extracts of DDGSPI: Lane 1-Albumin fraction; Lane 2- Globulin fraction; Lane 3-Prolamin fraction and Lane 4-Glutelin fraction; Lane 5-Molecular marker (10–200 kDa); Lane 6-DDGSPI.

Table 4
Amino acid composition of DDGSPI and glutelin.

Amino acid	Concentration (mg/g)		Relative amount (%)	
	DDGSPI	Glutelin	DDGSPI	Glutelin
Arginine	7.45	6.17	9.84	7.81
Histidine	2.02	1.94	2.66	2.45
Isoluecine	3.92	3.89	5.18	4.92
Leucine	7.2	6.99	9.51	8.84
Lysine	2.86	2.91	3.78	3.68
Threonine	3.83	3.56	5.06	4.5
Metheonine	2.46	2.88	3.25	3.64
Cysteine	0.41	0.33	0.54	0.41
Phenylalanine	4.66	4.79	6.15	6.06
Tyrosine	3.1	3.19	4.09	4.03
Valine	5.13	4.98	6.78	6.3
Aspartic acid	6.54	7.12	8.64	9.01
Glutamic acid	10.11	11.34	13.36	14.35
Serine	4.27	4.97	5.64	6.29
Proline	4.47	4.94	5.9	6.25
Alanine	2.94	3.59	3.88	4.54
Glycine	4.29	5.41	5.67	6.84

Essential amino acid	Standard protein (mg/mL)	Chemical score (%) DDGSPI	Glutelin
Lysine	55	68.73	67.00
Isoleucine	40	129.53	123.10
Leucine	70	136.00	126.40
Threonine	40	126.55	113.00
Valine	50	136.00	126.07
Phenylalanine + Tyrosine	60	171.00	168.35
Methionine + Cysteine	35	108.37	116.09

presence of globulin. In lane 3, three intense bands having molecular weight 42.5, 32.7 and 10.53 kDa were observed indicating the subunits of prolamin. Lane 4 is showing bands of glutelin subunits (32.7, 10.53 and 85 kDa). Lane 3 and 4 shows the presence of prolamin and glutelin which corroborates with the results reported by Sugimoto et al. (1986), Agboola et al., (2005) and Krishnan and Okita (1986). Similarly, lane 6 containing extracted DDGSPI shows 2 distinctive bands of MW 32.2 and 80.7 kDa. The result suggests that extracted protein isolates contains similar proteins of MW which is matching very close to the bands present in prolamin and glutelin fraction. A band of 10.11 kDa was also observed in lane 6 which corresponds to presence of albumin in extracted protein. Therefore, the obtained observations confirm the results depicted in Osborne method which indicated that glutelin and prolamin were the most abundant protein present in the substrate (Table 3).

3.9. Amino acid composition of DDGSPI and glutelin

Amino acid composition of a protein isolates is a measure of its nutritional value as shown in Table 4. It has been observed that, glutamic acid is a major amino acid present in DDGSPI and glutelin (13.36 % w/w and 14.35 %w/w, respectively). Arginine and leucine were the second most available amino acids. Aspartic acid, phenylalanine, isoleucine, proline, glycine, threonine and valine were also obtained significantly which comes under essential amino acids. In contrast, the amount of histidine was lowest in DDGSPI and glutelin i.e. 2.66 %w/w and 2.45 %w/w respectively, followed by metheonine and cysteine. Though availability of these essential amino acids is less in DDGSPI and glutelin, but they could be used as a complement with other food-based substrates which are rich in histidine and lysine to attain the proper nutritional balance. Moreover, the contents of hydrophobic and uncharged amino acids in glutelin and DDGSPI were approximately similar i.e. 47.39 %w/w and 46.32 %w/w, respectively as reported for groundnut protein isolate (Kudre et al., 2013).

Table 4 shows that the chemical score of the essential amino acid of DDGSPI and glutelin. Based on the amino acid profile, lysine % was found to be lowest in DDGSPI and glutelin, i.e approximately 69 %w/w and 67 %w/w, respectively. The chemical score of phenylalanine and tyrosine in DDGSPI (171.0 %w/w) and in glutelin (168.35 %w/w) were highest, followed by leucine, tyrosine, isoleucine, valine, methionine and cysteine which were higher than the recommended value of FAO/WHO requirements (Food and Agriculture Organization, 2007).

For feed/food formulations using protein isolates, it has been observed that during heating at high temperature, protein denaturation takes place which changes the nutritional and functional characteristics of the end product. The present study reveals that optimum extraction temperature for DDGS and WS is 70 °C. From the TGA graph, it can be interpreted that maximum degradation of biomolecules takes place above 130 °C. From this observation it can be deciphered that DDGS/WS proteins can retain its own 3-D structure up to 130 °C beyond which the denaturation takes place. Whereas, XRD data reveals that less crystallinity is present in the DDGS/WS proteins which means that it has tendency to mix with other nutrients and minerals easily as proteins (albumin, glutelin, prolamin and globulins) are globular in nature. FTIR data reveals the secondary structures of the DDGSPI and glutelin are intact and not denatured and thus, have proper nutritional contents for feed/food formulations.

4. Conclusion

Compositional, elemental and physical characterization of rice-based distillery byproducts helps in understanding that they could be used as a potential substrate in food formulation. Glutelin and prolamin were the major protein fraction available. A significant percentage of protein was extracted by 70% aqueous ethanol at 70°C in presence of 1% SMB. The maximum extracted protein was 47.7% (w/w) and 59.8% (w/w) with an increase in extraction fold by 1.45 and 2.0 for DDGS and WS, respectively. Thus, from the obtained results it can be inferred that, the maximum recovery of proteins has been achieved in the order of aqueous-ethanol > alkaline-ethanol > protease-mediated > ultrasonication > pH shifting techniques. The amino acid profiling helps to explore the possibilities of utilizing protein extracted from distillery byproduct in a better alternative way which could be directly utilized in food/feed formulation. In-addition SDS PAGE and SE-HPLC confirms the

identity of the proteins present in DDGS. Overall, the study establishes the potential feasibility of utilizing distillery proteins as a viable and attractive substrate for food industry.

Declaration of competing interest

There is no conflict of interest between the authors.

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