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Upgrading of valuable food component contents and anti-nutritional factors depletion by solid-state fermentation: A way to valorize wheat bran for nutrition

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ABSTRACT

Wheat bran is a by-product of wheat flour production used as a nutritious and economic feed ingredient, but since it possesses several anti-nutritional factors that entrap nutrients and interfere with digestion and absorption, its application is limited. The utilization of organic-rich wastes for the production of value-added products and nutritional improvement of food is a means to an end – sustainability. Here we propose addressing these challenges *via* fermentation by *Bacillus* sp. TMF–2, a strain that produces several enzymes and simultaneously, wheat bran of higher nutritional quality. Following solid-state fermentation, the soluble phenolic content of wheat bran nearly triplicated compared to the raw bran. The total proportion of polyphenols along with the antioxidant capacity and free radical scavenging rate were significantly improved, particularly the ability to reduce Fe^{3+} increased by 10-fold. Additionally, the activity of hydrolytic enzymes: amylase, cellulase, pectinase, mannanase, protease, and phytase increased during fermentation, whereas the level of anti-nutrient, phytic acid decreased. Among all produced enzymes, there is a special emphasis on the protease prone to degrade gluten thus resulting in gluten-free wheat bran. The results of this research could contribute to a beneficial procedure for the natural modification of wheat bran, which further augments the health benefits and utilization of wheat bran.

1. Introduction

The constant growth of population and increasing public awareness about environmental problems, on the one hand, imposed growth and development of food and agricultural industries, and on the other compelled each industry to reduce their footprint on earth resources. Hence, efficient utilization of resulting organic-rich wastes as a raw material for the production of value-added products has been an epicenter of scientific research for years. Additionally, the use of low-cost agro-industrial by-products and wastes as feed components reduces the feed cost, but in order to produce sustainable feed formulations, the supplementation of feed with exogenous enzymes has become common practice (Ravindran, 2013). Namely, the addition of exogenous enzymes to the feed enables increased digestibility of the indigestible components as well as the nutrients that are not being digested due to the association with the aforementioned compounds (Ravindran, 2013). Even though the addition of enzymes reduces the overall cost of the feed it would be much more lucrative to use agro-industrial wastes and by-products as the substrate for the microbial production of enzymes via solid-state fermentation, while simultaneously enabling the reduction of anti-nutritional factors. The solid-state fermentation (SSF), the process where microorganism grows in the environment without free water, has been used for thousands of years in food processing, but only recently it gained deserved attention in the production of value-added products (Salim et al., 2017). This technique although still innovative for commercialization, has numerous advantages: higher yields and productivities, extended stability of products, lower production cost, lower protein breakdown (which is especially important if an enzyme is the target product), lower contamination risk, and lower (or absent) catabolite repression (de Castro et al., 2018) over the industrially used

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submerged fermentation technique (SmF). SSF process requires significantly less energy input and is more economically feasible than SmF. Considering that SSF is firmly tied to the concept of valorization of agro-industrial waste through its use as culture media, significantly lower costs of raw materials and utilities than in SmF are expected. Furthermore, plant expenses corresponding to labor and downstream liquid waste treatment are reduced in SSF compared to SmF. The available literature data reveals that the unitary production cost of enzymes using SSF is about 3-fold lower than using SmF. For instance, the economic analysis indicated that the unit cost for cellulase production via SSF was $15.67/kg and $40.36/kg via SmF, while the corresponding market price for this enzyme was $90/kg (de Castro et al., 2010; Zhuang et al., 2007). Hence, the fermented agro-industrial waste has a competitive price since the production cost is 6-fold lower than the market price and it has favorable qualities such as the presence of desirable accessory enzymes and the absence of anti-nutritional elements. Additionally, environmental concerns caused by the traditional processes and search for alternative green and sustainable processes put solid-state fermentation into a new perspective.

Wheat bran is cheap lignocellulosic biomass that represents the main by-product of wheat flour production. Considering that the bran accounts for approximately 13–19% of wheat grain weight, it seems to perfectly serve as an abundant low-cost raw material for the production of functional food and feed ingredients (Onipe et al., 2015). Wheat bran is composed of the grain peripheral layers pericarp, testa, and aleurone layer, but it is noteworthy that during the grain milling the residual amounts of the starchy endosperm adhere to the bran components (Prückler et al., 2014; Rudjito et al., 2019). Generally, wheat bran comprises approximately of 12% water, 13–18% protein, 3.5% fat, and 56% carbohydrates (Prückler et al., 2014). Around 55% of the dietary fiber in wheat bran is arabinoxylan and the rest is cellulose (9–12%), lignin (3–5%), fructan (3–4%), and mixed linked β-glucan (2.2–2.6%). About 95% of the dietary fiber in wheat bran is insoluble (Sibakov et al., 2013). Wheat bran has higher antioxidant potential compared to other grain constituents due to the high concentration of secondary metabolites such as flavonoids, and phytosterols, but mostly this phenomenon is attributed to the higher concentrations of phenolic acids (Singh et al., 2012). Phenolic acids are divided into two categories, derivatives of hydroxybenzoic and hydroxycinnamic acid (Kim et al., 2016). Hydroxycinnamic acids are more prevalent in wheat bran with ferulic acid being the most abundant and accounting for approximately 85% of total phenolic acids (Zhang et al., 2014; Kim et al., 2016). Additionally, wheat bran is rich in dietary fiber and minerals (Prückler et al., 2014), but its nutritional value is adversely affected by the high amount of antinutritional factors that could be overcome by reducing their levels as a consequence of the metabolic activity of microorganisms during solid-state fermentation.

Hence, the main idea behind this work was to create a sustainable feed component by using the low-cost by-product abundant in Serbia and simultaneously produce a wide range of enzymes via solid-state fermentation. Sustainable feed component was enhanced by bacterial strain isolate *Bacillus* sp. TMF–2 in an attempt to ameliorate the antioxidant potential and nutritional quality of wheat bran. Concurrently, the potential of wheat bran as a substrate for co-production of the multienzyme complex has been considered.

2. Materials and methods

2.1. Sample materials and chemicals

The wheat bran (protein ($N \times 6.25$) 18.03 ± 0.04% dry basis, ash 3.93 ± 0.05% dry basis, crude cellulose fiber 6.87 ± 0.021% dry basis) used in this study was obtained by processing winter wheat Serbian cultivar Simonida, in a local mill sited in Čenta (Serbia). The Simonida cultivar (*Triticum aestivum L.* ssp. *vulgare* var. *Lutescens*) is red-grained hard wheat variety characterized with white and smooth spike and it belongs to the A–2 quality subgroup. The substrate was dried overnight in the vacuum oven (Vacuum oven VD23, Binder, Tuttlingen, Germany) at 60 °C, ground using a ball mill (Mixer Mill MM 500, Retsch, Haan, Germany) and sieved to obtain substrate with particle size between 200 and 800 μm. The sieved substrate was kept in a desiccator until usage.

The reagents used to determine phenol content and antioxidant activity: Folin–Ciocalteu reagent, TPTZ (2,4,6-tripyridyl-s-triazine), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and gallic acid were all purchased from Sigma–Aldrich (St. Louis, USA). All of the substrates used for determination of enzyme activities: Azo-casein for protease, pectin from apple for pectinase, carboxymethylcellulose for cellulase, starch for amylase, and Locust bean gum from *Ceratonia siliqua* seeds for mannanase, were also obtained from Sigma–Aldrich (St. Louis, USA). Sodium phytate used to determine phytase activity and content of phytate in the bran was also purchased from Sigma–Aldrich (St. Louis, USA). All other chemicals and solvents were of the highest commercial grades purchased from Merck (Darmstadt, Germany) and Lach-Ner (Bratislava, Czech Republic).

2.2. Strain screening and identification

Myriad isolates belonging to the diverse Collection of cultures of the Faculty of Technology and Metallurgy, University of Belgrade were screened for extracellular enzymatic activities using selective agar plates as previously described by Salim et al. (2017). Namely, amylolytic activity was assessed using starch-based agar plate, for cellulolytic activity-CMC agar plate was employed, mannanase activity was screened by observing hydrolysis halos on a mannan containing-LB agar plate and for pectinase activity-pectin agar plate was employed. The ability of the tested microorganism to degrade gluten was estimated using the agar plate method as described previously by Stressler et al. (2015).

Amongst tested strains, one strain isolated from spoiled dairy and milk products stood out, as it showed promising activities on every agar plate including gluten agar plate for which it was unique amongst others that were tested. This strain, named *Bacillus* sp. TMF–2, was therefore selected for further studies. The strain was identified as *Bacillus* sp. based on the methods reported in Bergey's Manual of Determinative Bacteriology, which was later confirmed through molecular characterization of 16 S rRNA gene sequence.

The "Quick-DNA Fungal/Bacterial Miniprep Kit" kit (Zimo Research) was used to isolate genomic DNA from bacteria. Isolated genomic DNA is further used for gene amplification by polymerase chain reaction (PCR). The universal primers UN116SF 5′-GAGAGTTTGATCCTGGC-3′ and UN116SR 5′- AGGAGGTGATCCAGCCG -3′ were used in the PCR reaction (Dimkić et al., 2013). PCR amplification was performed in 0.05 ml of the reaction mixture containing 0.001 ml of DNA, 0.025 ml of DreamTaq PCR Master Mix (Thermo Scientific), 0.5 μM of each primer, and nuclease-free PCR water supplemented to 0.05 ml. On the 'T100 Thermal cycler' Biorad™, PCR reactions were performed according to the following protocol: initial denaturation at 95 °C for 3 min, 40 cycles consisting of denaturation at 95 °C for 15 s, primer hybridization at 53 °C for the 30 s and 72 °C extensions for 90 s, and final extension at 72 °C for 90 s. The length of the PCR products was determined by horizontal electrophoresis (20 min at 100 V) at 2% agarose. PCR products were purified via the 'DNA Clean & Concentrator™' kit (Zymo Research) and sent for sequencing to the "Macrogen" sequencing service (Netherlands). PCR product sequences were analyzed using the BLAST nucleotide sequence search program on the National Center for Biotechnology Information (NCBI) website.

2.3. Solid-state fermentation

The 150 ml Erlenmeyer flasks containing the medium, wheat bran, were tightly plugged with cotton and autoclaved at 121 °C and 0.12 MPa for 20 min. Upon cooling, 0.5 ml of bacterial suspension (24 h bacterial culture in LB medium, ~1 × 10^6 cells/ml) was aseptically added to the wheat bran in the flasks, along with the sterile distilled water for the moisture content of 1:1 solid to liquid ratio. All samples were incubated statically at 30 °C for up to 11 days. Each day of fermentation samples were taken to determine the kinetics of the enzyme production, and change in gluten, phytic acid and phenol content. Sterilized wheat bran without bacteria was used as a control sample. All measurements were conducted in triplicate. At the end of each fermentation, extraction of the enzymes was performed by the addition of distilled water so that liquid:solid ratio of 5 ml/g was achieved. Samples were then mixed on a rotary shaker (KS 4000 i control, IKA, Staufen, Germany) for 30 min at 30 °C and 180 rpm. This was followed by centrifugation (Heraeus™ Fresco™ 17 Microcentrifuge, The Thermo Scientific, Waltham, USA) of the contents at 8000 rpm for 10 min (4 °C). The resulting supernatants were considered as a crude enzyme extract and evaluated for the activities of protease, cellulase, α-amylase, pectinase, mannanase, and phytase. The residue after centrifugation was dried in the vacuum oven for 48 h at 30 °C and afterward stored at 4 °C until further experiments. Additionally, the microbial growth was monitored by plating the suitably diluted extracts on LB agar and expressed as a number of colony forming units after 72 h of plate incubation at 30 °C.

2.4. Enzymatic assays

2.4.1. α-Amylase, pectinase, cellulase, and mannanase activity assays

The activities of α-amylase, pectinase, cellulase, and mannanase were measured according to the levels of reducing sugars liberated from the soluble starch, pectin, carboxymethyl cellulose, and locust bean gum, respectively. The reducing sugars liberated were estimated by the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959). The activities were determined using calibration curves for maltose, galacturonic acid, glucose, and mannose, respectively. One unit of enzyme activity was defined as the liberation of appropriate reducing sugar equivalent to 1 μmol/min under the assay condition according to the DNS method. Assay conditions were previously reported in detail by Salim et al. (2017).

2.4.2. Phytase activity determination

Phytase activity was determined using Sigma Aldrich Enzymatic Assay of Phytase (EC 3.1.3.8), which is based on the colorimetric determination of inorganic phosphate liberated in the reaction between phytase and phytic acid. The method was modified only in the volume of enzyme used in the assay since we were using fermentation extract and not purified enzyme. Therefore, instead of 0.025 ml as assay suggests we used 0.2 ml of crude enzyme extract. One unit of phytase activity is defined as the amount of enzyme that will liberate 1.0 μmol of inorganic phosphorus from 0.0315 M phytate per minute at assay conditions (pH 2.5 at 37 °C). The standard curve for phosphorus determination was created using potassium phosphate and sodium phytate as the assay suggests.

2.4.2. Protease activity assay

The proteolytic activity was measured using azo-casein as a substrate and it was previously described by Salim et al. (2017). One unit of proteolytic activity was defined as the quantity of enzyme that produced a unitary difference in absorbance at 440 nm between the reaction blank and the sample under the assay conditions.

2.5. Phytic acid and inorganic phosphorus determination

Bran phytate was measured according to the colorimetric method based on the disappearance of the pink color of Wade reagent in the presence of phytate. Samples of unfermented and dried fermented wheat bran, 0.5 g each, were extracted with 20 ml of 2.4% HCl for 2 h with a magnetic stirrer at room temperature (Coelho et al., 2002). The extracts, 2 ml, were centrifuged (MiniSpin®, Eppendorf, Hamburg Germany) at 5000 rpm for 4 min. Afterward, an aliquot of 0.5 ml of each centrifuged extract was diluted by mixing with 14 ml of water and 0.5 ml of 0.11 M Na_2EDTA +0.75 M NaOH (Gao et al., 2007). The diluted extract was passed through a column (0.7 × 15 cm) packed with anion exchange resin (Amberlite® IRA-400 chloride form). Inorganic phosphorus was eluted with 15 ml of 0.1 M NaCl and determined according to the method reported by Bencini et al. (1983) using anhydrous potassium phosphate as a standard. Phytate was eluted with 15 ml of 0.7 M NaCl and determined using Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ + 0.3% sulfosalicylic acid). Namely, 0.9 ml of appropriately diluted eluent was mixed with 0.3 ml of Wade reagent and the absorbance was read at 500 nm on a spectrophotometer (UV/Vis Ultrospec 3300 Pro, Amersham Bioscience, UK). A series of calibration standards containing 5–40 μg/ml were prepared from sodium phytate. The results are presented using phytic acid degradation rate (PAD) which is calculated as proposed by Zhao et al. (2017).

2.6. Determination of total phenols

Total phenolic content (TPC) was analyzed according to Folin–Ciocalteu method with small modifications (Pavlović et al., 2013). Fast microwave-assisted extraction of phenolics with 20% aqueous ethanol was used, as previously reported by Pavlović et al. (2013) for spent coffee. The method was conducted with small changes, namely, 1 g of each sample was extracted using 15 ml of 20% aqueous ethanol under just 70 s of microwave radiation 300 W (LG MC7849HS). Afterward, extracts were centrifuged (Sigma 2-16 P, Sigma, Osterode am Harz, Germany) at 8000 rpm for 3 min. The percentage of dry matter in the extracts was measured on a moisture analyzer (Kern MLS-A, Balingen, Germany). Further, 0.05 ml of the extract was mixed with 0.25 ml Folin–Ciocalteu reagent and 3.7 ml of distilled water and 1 ml of 15% Na_2CO_3. This mixture was kept in dark at room temperature for 2 h and afterward, absorbance was read at 750 nm. TPC was expressed as equivalents of gallic acid per gram of dry wheat bran.

2.7. Determination of antioxidant activity

The extracts obtained for TPC analysis were further used to determine their antioxidative potential using different methods. Prior to analysis, appropriate dilutions of samples were made using 20% aqueous ethanol.

2.7.1. Determination of DPPH scavenging activity

The determination of DPPH activity of the extracts was assessed using two different approaches, fixed reaction time set at 30 min, and steady-state measurement. For the steady-state measurement, 0.18 ml of 0.1 mM methanolic DPPH radical solution was mixed with 0.02 ml appropriately diluted extract (1–5 mg/ml), and decrease in absorbance was monitored at 517 nm for 90 min (all extracts reached steady state) using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific™, Waltham, United States). Obtained results were presented as EC_{50} values i.e. effective concentrations of extract necessary to decrease the initial absorbance of radical by 50% Mishra et al. (2012). The EC_{50} was calculated from the plot presenting the dependency of the percentage of the remaining radical (Mishra et al., 2012; Savatović et al., 2012) on the mass ratio of the extract to DPPH radical. Measurement conducted using fixed reaction time was done according to the same experimental procedure, but inhibition of DPPH radical was measured only at one time, after 30 min. Obtained results, in this occasion, were calculated as IC_{50} values, i.e. minimal inhibitor concentrations that were calculated according to the aforementioned literature (Mishra et al., 2012; Savatović et al., 2012). The reaction temperature was set at 15 °C in order to reduce methanol evaporation.

2.7.2. Determination of ABTS scavenging activity

The determination of $ABTS^{\bullet+}$ radical scavenging activity of extracts was conducted using the method previously described in detail by Elmalimadi et al. (2017). Briefly, an aliquot of 1.0 ml of diluted ABTS radical cation solution (produced in the reaction between 7 mM ABTS solution and 140 mM potassium persulfate performed in dark for 12–16 h) was mixed with 0.01 ml of diluted extract (1–5 mg/ml) incubated at room temperature for 5 min and the change in absorbance against PBS buffer, which was used for the preparation of ABTS radical cation solution, was monitored at 734 nm. Results were expressed as a minimal inhibition concentration, IC_{50} of extract needed to achieve inhibition of 50% of the radical.

2.7.3. Determination of ferric reducing antioxidant power-FRAP method

Determination of ferric reducing antioxidant power was assessed using the FRAP method previously described (Pavlović et al., 2013), with small modifications. Namely, 0.03 ml of extract (1–5 mg/ml) was mixed with 0.9 ml of FRAP solution prepared as described by Pavlović et al. (2013). Results were expressed as the Trolox equivalents per g of substrate.

2.8. Gluten quantification

Sample preparation for gluten quantification was as follows: 1 g of a dried homogeneous sample was extracted with 10 ml of 60% ethanol and afterward, gluten quantification was conducted using a competitive ELISA, RIDASCREEN® Gliadin competitive (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

2.9. HPLC analysis

HPLC analysis of carbohydrates was performed using Dionex Ultimate 3000 Thermo Scientific system (Waltham, MA, USA) with Hyper REZ XP (Carbohydrate Ca^{2+}, 300 mm × 7.7 mm, 8 μm) column and RI detector (RefractoMax 520, ERC, Riemerling, Germany). The method lasted for 25 min with a flow of 0.6 ml/min and a column temperature of 80 °C. An injection volume of samples was 0.05 ml.

2.10. Statistical analyses

The experimental results are presented as the mean ± standard deviation of the three technical repeats. One-way ANOVA followed by Tukey's test was used to identify differences among means. The statistical significance level was set to 0.05. Pearson correlation coefficient was used to examine the relationship between the studied parameters, $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered as highly significant. Statistical analyses were performed using Microsoft Excel 365 and Origin 8.

3. Results and discussion

3.1. Kinetics of enzyme production

Utilization of agro-industrial by-products and wastes as raw materials for the production of value-added products has been an engrossing matter since these substrates nutritionally meet the needs for microbial growth, while simultaneously being improved in the aspect of their nutritional features. However, in order to use them efficiently, microorganisms need to produce extracellular enzymes that would degrade building macromolecules of which these agro-industrial wastes are composed. Therefore, in order to find adequate microorganism that would enable efficient utilization of lignocellulosic waste materials, we have conducted screening to find the one that produces extracellularly different hydrolytic enzymes. A variety of microorganisms isolated from soil and food samples and kept as a part of Collection of cultures of the Faculty of Technology and Metallurgy, University of Belgrade was screened using different selective agar plates. One strain, *Bacillus* sp. TMF–2, isolated from spoiled dairy and milk products stood out as an efficient producer of α-amylase, mannanase, cellulase, and pectinase since it showed halo zones (hydrolysis zone greater than 2 cm) on agar plates (Supplementary material, Fig. S1). Additionally, this strain showed the potential for gluten degradation. The microscopic examination of the isolate showed that it was a Gram positive rod-shaped spore-forming microorganism and the appearance of colonies on solid medium (flat white opaque colonies with a smooth surface and irregulary shaped margins) implied that the isolate most likely was a member of *Bacillus* sp. This was further confirmed via 16 S rRNA gene sequence analysis. The resulting gene sequence, ~1500 bp, was analyzed with Blast through the NCBI server and the results show that the strain belongs to the *Bacillus* genus, more precisely *Bacillus subtilis* subgroup and that it produces equal degrees of similarity to sequences from several *Bacillus* species (5 species with more than 99%) and therefore sequencing of 16 S rRNA gene could not provide an adequate identification on the species level.

Since wheat has been widely cultivated in Serbia, in the present study wheat bran was chosen as the substrate for solid-state fermentation. The activities of α-amylase, cellulase, pectinase, and mannanase were monitored throughout the fermentation and the results are presented in Fig. 1.

The time course of the α-amylase activity during fermentation presented in Fig. 1 shows sharp activity increase reaching its maximum value of 107 IU/g on the 7th day of fermentation after which the decline in activity is observed. The reason for this behavior could be related to the kinetics of bacterial growth since the microbial growth follows the same trend, as presented in Fig. 1. Namely, due to the fact that most of the nutrients are not readily available in the medium, the growth curve does not show typical phases. After the initial exponential growth phase, the viable cell count seems to remain constant until the 5th day of the fermentation. Concomitantly with the observable increase in the production of enzymes, suspected changes in nutrient availability facilitate further microbial growth thus a shifted increased stationary growth phase is observable up to the 10th day of fermentation. The sharp decrease in the amylase activity is related to the starch location in the bran. Namely, even though wheat bran mostly comprises of non-starch polysaccharides, starch granules are located in the aleurone layer and they originate from the starchy endosperm which adheres to the aleurone layer of bran after the dry milling process (Rudjito et al., 2019; Yin et al., 2018). Yin et al. (2018) recently reported that fungi degrade wheat bran starting with the aleurone layer and therefore the high activity of amylase correspondent to the microbial growth is expected. Additionally, a sharp decrease in the activity can be attributed to the

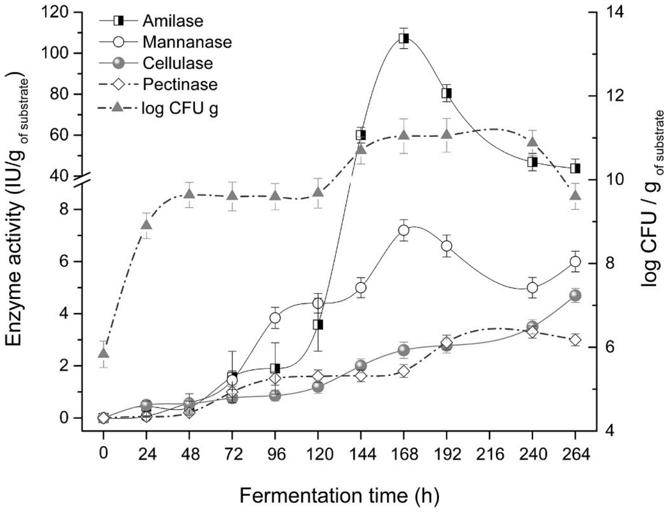

Fig. 1. Kinetics of microbial growth and enzyme production on wheat bran using *Bacillus* sp. TMF–2. The poined data present mean of three technical repeats ± SD ($n = 3$).

degradation of available starch in the aleurone layer. The same trend can be observed for the mannanase activity, only with a much lower yield. The reason is the very low amount of glucomannan in wheat bran, which originates from the aleurone and endosperm cells (Chateigner-Boutin et al., 2014; Schooneveld-Bergmans, 1997). Due to the location of these small amounts of glucomannan, a similar trend with amylase activity is expected and Pearson's correlation coefficient reveals strong positive interaction (Table 2). The highest activity of mannanase is obtained after 7 days of fermentation when the value of 7.2 IU/g is achieved. Afterward, the decline in activity is noticeable, but to a much lesser extent compared to the amylase activity. Namely, some researchers suggest that wheat cell wall components are not only an association between arabinoxylans and mixed-linked glucans, but more complex structures containing, amongst other polymers, some amount of mannans (Chateigner-Boutin et al., 2014).

Results obtained for cellulase activity show that the enhanced production of cellulase happens later on in the fermentation even though some activity is observed in the early stages of fermentation. Cellulose represents one of the major non-starch polysaccharides in wheat bran which is mostly located in the pericarp, but some amount is present also in the intermediate layer as well as in aleurone (Schooneveld-Bergmans, 1997; Yin et al., 2018). For this reason, the secretion of cellulolytic enzymes is induced with a higher degree of efficiency later on in the fermentation after the aleurone layer has been biodegraded. The highest activity (4.7 IU/g) is achieved after 11 days of fermentation. Pectinases are obtained with the lowest yield with the maximum activity of 3.3 IU/g after 10 days of fermentation. The existence of pectin in wheat grain has been recently proved by a group of authors. They showed that rhamnogalacturonan I is located mostly in the endosperm and aleurone layer, while homogalacturonan was mainly located in the outer layers (Chateigner-Boutin et al., 2014). Since wheat bran comprises of aleurone and outer layers of wheat grain, both of the pectic polysaccharides found in wheat are expected to be present in the bran. The kinetic results suggest that the pectinase produced by this strain has more affinity towards homogalacturonan since they are secreted later in the fermentation (Chateigner-Boutin et al., 2014; Rudjito et al., 2019).

3.2. *Phytic acid degradation rate and phytase production*

Besides the production of enzymes, the solid-state fermentation of wheat bran is beneficial from the viewpoint of the nutritional quality of bran, making it more suitable as a feed ingredient due to the enhanced nutrient bioavailability. Namely, even though wheat bran presents a very cheap but nutritious feed component, a high content of anti-nutritional factors adversely affects its utilization in animal digestion, with phytic acid being one of them. Results obtained in this study show that phytic acid is partially degraded during the fermentation of wheat bran with *Bacillus* sp. TMF–2. The degradation rate had a gradual increase as the fermentation proceeded and reached a maximum level of ~34%, after which no further increase was observed (Fig. 2). Even though there are two enzymes present in wheat that can hydrolyze phytic acid, the autoclaving of wheat bran before fermentation inhibited the activity of these endogenous phytases (Zhao et al., 2017). Hence, the degradation of phytic acid seems to be a consequence of microbial phytase or phosphatase produced by *Bacillus* sp. TMF–2. To confirm this assumption, the phytase activity was measured each day of fermentation and we observed that the activity increased as the fermentation proceeded. Literature data corroborate our results since *Bacillus* strains are well documented as the phytase producers (Kumar et al., 2016).

The time course of phytase production, phytase acid degradation rate, and inorganic phosphorus release in solid-state fermentation using wheat bran as the substrate is shown in Fig. 2. As shown, the phytase activity had a gradual increase until day 4 of fermentation after which further increase was negligible. The corresponding trend was observed for the phytic acid degradation rate and release of inorganic phosphate, which is the product of phytic acid hydrolysis.

Presented results confirm that the working strain produces phytase that can efficiently hydrolyze phytic acid, making the fermented wheat bran a very promising component of animal feed. Namely, 60–90% phosphorus in cereals is found in the form of phytic acid, and as such unavailable for swine, poultry, and pre-ruminant calves, which do not have phytic acid hydrolyzing enzymes in the gastrointestinal tracts (Kumar et al., 2016; Singh et al., 2011). This represents the problem from several viewpoints. Firstly, unused phosphorus from animal feed represents a serious water pollution problem since it leads to water eutrophication (Singh et al., 2011). Further, phytic acid can reduce the availability of positively charged nutrients through chelation and also acts as an inhibitor of some digestive enzymes (Kumar et al., 2016; Singh et al., 2011). Additionally, phosphorus supplementation in animal feed represents serious cost and it would be much more lucrative if phosphorus already present in feed could be more efficiently utilized (Kumar et al., 2016). Keeping this in mind, an increase in available phosphorus of wheat bran produced during phytic acid hydrolysis, as presented in Fig. 2, represents an additional benefit for the application of fermented wheat bran as an animal feed component.

Dephytisation of wheat bran via fermentation with lactic acid bacteria with a similar degradation rate of 27% was recently reported (Zhao et al., 2017). Other authors reported complete phytic acid hydrolysis in

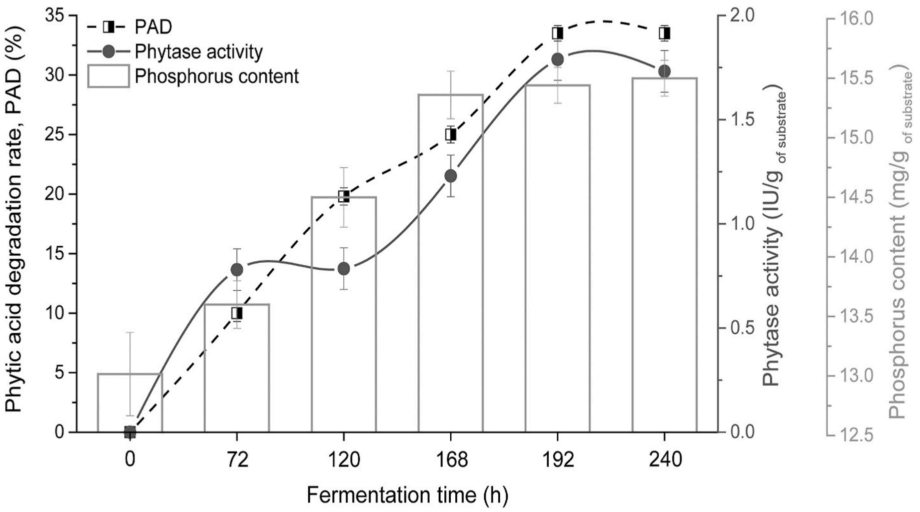

Fig. 2. The time course of phytase production, phytic acid degradation rate (PAD) and inorganic phosphorus release during solid-state fermentation of wheat bran with *Bacillus* sp. TMF–2.

wheat bran (Manini et al., 2014). Optimization of phytase production using wheat bran as a substrate with *Bacillus* sp. TMF–2 in solid-state fermentation would probably lead to higher phytic acid degradation rates since it was shown as a promising phytase producer.

3.3. Phenolic compounds and antioxidative potential of fermented wheat bran

The phenolic compounds were microwave-extracted using 20% aqueous ethanol as solvent. This method was previously proved as efficient for the extraction of phenolic compounds from spent coffee (Pavlović et al., 2013). The method is eco-friendly and food safe which is of great importance since the fermented wheat bran is intended for animal consumption.

Results obtained for total phenolic content for wheat bran and fermented wheat bran are presented in Table 1. The total phenolic content of unfermented wheat bran was found to be 1820 μg GAE/g, which was in good agreement with the literature data (Yin et al., 2018). On the other hand, Zhao et al. (2017) reported much higher values for unfermented wheat bran. The discrepancies in the obtained results could be attributed to the wheat varieties and different extraction solvents and conditions. In general, alcohol/water solutions were proved better for the extraction of phenolics than mono-component solvents due to the solubility of phenolic compounds (Bhanja Dey and Kuhad, 2014).

It is noticeable from the results, that a slight decrease in extracted phenolics occurs after autoclaving, probably due to the degradation of phenolic compounds at high temperatures (Zhao et al., 2017). As the fermentation proceeds, the phenolic content increases continuously reaching 5730 μg GAE/gafter 10 days of fermentation. The increasing phenolic content is in accordance with the microbial growth. The increased phenolic content after fermentation was already reported in several other studies (Zhao et al., 2017; Yin et al., 2018). The release of phenolic compounds during fermentation is associated with the degradation of cell wall materials since most of the phenolics in cereals are found in bound form (Zhang et al., 2014). More specifically, the phenolic acids in wheat bran are found esterified or covalently bound to cell wall polysaccharides (Kim et al., 2016; Yin et al., 2018). Due to the complex structure of these cell wall polymers, a synergistic action of several hydrolytic enzymes is necessary to release these phenolic compounds. Presence of hemicellulases especially xylanases, β-xylosidase, and α-arabinofuranosidase is important since wheat bran predominantly comprises of non-starch polysaccharides with arabinoxylans being the predominant ones (Sorensen et al., 2003). Enzymatic digestion of these compounds depends on the synergetic effect of hemicellulases with feruloyl esterases, which cleaves ester bonds between arabinoxylans and phenolic acids, as well as two arabinoxylan chains, and therefore eases the access of hydrolases to the backbone of polymer (Hell et al., 2015; Mathew and Abraham, 2004). The strain used in this study has already been proved as a good producer of amylase, cellulase, and pectinase while the profile of monosaccharides of fermented wheat bran compared to raw wheat bran points out to the conclusion that this strain also produces hemicellulase. Namely, after fermentation distinct peaks of xylose and arabinose appeared (Fig. 3), which may be indicative of hemicellulase catalyzed degradation of cell wall polysaccharides. Additionally, the results presented in Fig. 3 are in accordance with the microbial growth. The synergistic effect of these enzymes enabled efficient degradation of biomass which resulted in a 3.3-fold increase in soluble phenolic content. The values of Pearson's coefficients presented in Table 2 reveal that all of the enzymes show strong or very strong correlation with total phenolic content.

Table 1
Total phenolic content and antioxidant activity expressed as EC_{50} and IC_{50} values for DPPH$^{\bullet}$ scavenging activity, IC_{50} value for ABTS$^{\bullet+}$ scavenging activity and nmol TE/g grain for FRAP for unfermented and fermented wheat bran after different fermentation time.

Fermentation time (h)	Total phenolics (μg GAE/g)	EC_{50} of DPPH$^{\bullet}$ (mg/ml)	IC_{50} of DPPH$^{\bullet}$ (mg/ml)	IC_{50} of ABTS$^{\bullet+}$ (mg/ml)	nmol TE/g grain
Control bran	1820 ± 27.3^{ab}	6.42 ± 0.077^{a}	7.02 ± 0.126^{a}	6.60 ± 0.139^{a}	53 ± 1.06^{a}
Autoclaved control bran	1570 ± 47.1^{a}	6.57 ± 0.084^{a}	7.45 ± 0.089^{b}	6.62 ± 0.106^{a}	45 ± 0.85^{a}
Fermented bran 24	1920 ± 48.0^{b}	6.23 ± 0.093^{b}	7.05 ± 0.099^{a}	6.02 ± 0.054^{b}	82 ± 0.78^{b}
Fermented bran 48	1610 ± 28.98^{a}	7.25 ± 0.065^{c}	9.11 ± 0.082^{c}	6.08 ± 0.079^{b}	74 ± 1.04^{b}
Fermented bran 96	2980 ± 134.1^{c}	4.50 ± 0.090^{d}	5.22 ± 0.045^{d}	4.01 ± 0.070^{c}	257 ± 4.63^{c}
Fermented bran 168	4380 ± 87.6^{d}	2.82 ± 0.045^{e}	2.91 ± 0.061^{e}	2.81 ± 0.024^{d}	420 ± 5.67
Fermented bran 192	5680 ± 93.72^{e}	1.63 ± 0.044^{f}	2.73 ± 0.045^{ef}	1.97 ± 0.026^{e}	580 ± 10.32^{e}
Fermented bran 240	6002 ± 180.6^{f}	1.87 ± 0.018^{g}	2.13 ± 0.038^{g}	2.78 ± 0.035^{d}	578 ± 5.49^{e}
Fermented bran 264	5730 ± 160.44^{ef}	2.30 ± 0.028^{h}	2.69 ± 0.023^{f}	1.58 ± 0.015^{f}	527 ± 6.48^{f}

Values are mean of technical repeats ± SD ($n = 3$). Data within a column with different lowercase superscript letters are significantly different according to Tukey's test ($P < 0.05$).

The antioxidant activity of wheat bran and fermented wheat bran was assessed using three methods, scavenging activity of two radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ and the FRAP method. Results obtained by the FRAP method are expressed as Trolox equivalents/g of wheat bran or fermented wheat bran, while DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities are expressed as concentrations of extract needed to achieve inhibition of 50% of the radical. Inhibition of DPPH$^{\bullet}$ radical is a widely used method to evaluate the antioxidant potential of the extracts, but the commonly used method with measurement of inhibited DPPH$^{\bullet}$ radical at a fixed time (usually 30 min) has flaws since there is a significant difference in the kinetic behavior of disappearance of DPPH$^{\bullet}$ radical in presence of different antioxidants (Mishra et al., 2012). The time required to achieve maximum scavenging of DPPH$^{\bullet}$ radical differs depending on the concentration and type of antioxidant (Mishra et al., 2012), and therefore we have conducted scans at different time intervals so that each extract concentration can reach maximum scavenging of DPPH$^{\bullet}$ radical. The results obtained during these scans show that extracts take more than 30 min to reach the steady-state and according to the antioxidant classification, proposed by Mishra et al. (2012), they can be classified as the ones with slow kinetics. Basically, antioxidants with fast kinetics reach a

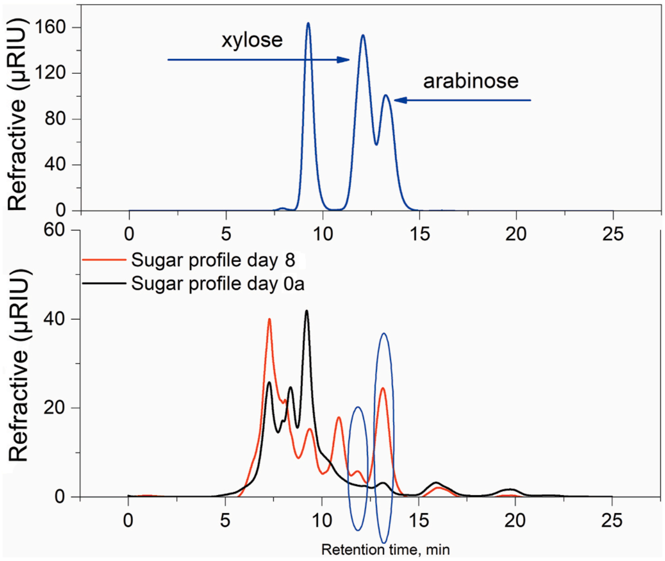

Fig. 3. Chromatogram of extracts obtained on the 8th day of wheat bran fermentation with *Bacillus* sp. TMF–2 and autoclaved raw wheat bran.

Table 2
The correlation matrix between all measured parameters, enzyme activities, total phenolics, and antioxidant activity expressed as EC_{50} and IC_{50} values. The highly significant interactions are colored red ($P < 0.01$), significant interactions are colored orange ($P < 0.05$) and factors with no significant interaction are colored blue.

	Total phenolics	EC_{50} DPPH							
EC_{50} DPPH	−0.980	1.000	IC_{50} DPPH						
IC_{50} DPPH	−0.956	0.987	1.000	EC_{50} ABTS					
EC_{50} ABTS	−0.956	0.963	0.939	1.000	FRAP				
FRAP	0.995	−0.988	−0.958	−0.966	1.000	α-amylase			
α-amylase	0.733	−0.791	−0.785	−0.764	0.765	1.000	Mannanase		
Mannanase	0.887	−0.934	−0.921	−0.958	0.917	0.879	1.000	Cellulase	
Cellulase	0.941	−0.885	−0.875	−0.923	0.915	0.666	0.826	1.000	Pectinase
Pectinase	0.986	−0.965	−0.935	−0.953	0.986	0.657	0.876	0.916	1.000

steady state in less than 30 min, a medium between 30 min and 1 h, while those with slow kinetics need more than 1 h to reach a steady-state (Mishra et al., 2012). Due to this observation, results are expressed using two values, EC_{50} and IC_{50}. The EC_{50} value refers to the steady-state kinetics and it represents the concentration of an antioxidant needed to decrease the initial absorbance of the DPPH$^{\bullet}$ radical by 50% (Mishra et al., 2012). In order to calculate this value percentage of remaining DPPH$^{\bullet}$ radical after each extract concentration reached steady-state is plotted against the mass ratio of extract to DPPH$^{\bullet}$ radical. The IC_{50} value was calculated for each extract and it represents the concentration of the extract necessary to scavenge 50% of the DPPH$^{\bullet}$ radical in 30 min. Antioxidants with fast kinetics have the same values for the EC_{50} and IC_{50} since they reach steady-state in less than 30 min (Savatović et al., 2012). On the other hand, extracts obtained in this study have different values for calculated EC_{50} and IC_{50} as is expected for antioxidants with slow kinetics (Table 1.), This is probably due to the fact that some phenolic compounds found in wheat are classified as intermediate or slow kinetic antioxidants (Mishra et al., 2012). Keeping this in mind the presentation of only IC_{50} value of the extract obtained at fixed reaction time would diminish the real antioxidant potential of the extract towards the DPPH$^{\bullet}$ radical. For comparison we have determined EC_{50} and IC_{50} values for Trolox, phenolic synthetic antioxidant, and determined that it follows fast kinetics in the reaction with DPPH$^{\bullet}$ radical, hence EC_{50} and IC_{50} are equal and their value is 50 μg/ml. In comparison with Trolox, the extracts do not show enviable antioxidative potential towards DPPH$^{\bullet}$ radical, but it is noticeable that fermented wheat bran has much greater antioxidant potential compared to raw wheat bran. This result is in accordance with the results for total phenolic content, hence antioxidant activity is phenolic content related, with Pearson's coefficient −0.980 and −0,956 for the EC_{50} and IC_{50} of DPPH$^{\bullet}$ radical assay respectively.

It is noticeable that autoclaved unfermented wheat bran has a bit lower antioxidant potential compared to unfermented wheat bran, probably due to the already mentioned degradation of phenolic acids at high temperatures (Zhao et al., 2017). The EC_{50} and IC_{50} values of DPPH$^{\bullet}$ radical assay for unfermented and autoclaved wheat bran were 6.57 and 7.45 mg/ml respectively, which was in good agreement with the results obtained by Bhanja Dey and Kuhad (2014) who showed that raw wheat bran has an IC_{50} value of 5.25 mg/ml. The highest antioxidant potential measured by the DPPH method was observed after 8 days of fermentation with EC_{50} being 1.63 mg/ml. Afterward, EC_{50} values have begun to decrease even though TPC increased slightly. The reason for this phenomenon could be attributed to the microbial biodegradation of phenolic acids. Namely, some *Bacillus* strains can transform ferulic acid and *p*-coumaric, which are the most abundant phenolic acids in wheat (Kim et al., 2016), to vanillin and *p*-hydroxybenzoic acid (Peng et al., 2003; Zhang et al., 2014). This can affect the overall antioxidant capacity of the extract since the presence of the CH═CH–COOH group in the cinnamic acid derivatives enables higher antioxidant efficiency of these compounds compared to hydroxybenzoic derivatives (Kim et al., 2016; Zhang et al., 2014). Bhanja Dey and Kuhad (2014) reported an IC_{50} value of 0.64 mg/ml for wheat bran fermented with *Rhizopus oryzae* RCK2012. The higher antioxidant capacity can be a consequence of different phenolics released during fermentation due to the differences in the produced enzymes. Additionally, the hyphal mode of growth gives an advantage to filamentous fungi over the bacteria in the utilization of solid substrates (Zhang et al., 2014).

The antioxidant capacity of extracts of wheat bran and fermented wheat bran was also assessed by their capability to scavenge ABTS$^{\bullet+}$ radical. The results are very similar to the ones obtained by the DPPH method. The IC_{50} value of wheat bran 6.6 mg/ml decreased significantly during the fermentation reaching the value of 1.58 mg/ml after 11 days of fermentation. The discrepancies in the trend obtained by the ABTS method compared to the DPPH method could be ascribed to the differences in solubility of the obtained extracted phenolics since different solvents (methanol and PBS buffer) are used in these methods (Zhang et al., 2018). Additionally, different mechanisms are ascribed to the radical scavenging of these radicals. Even though quenching of both DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals occurs through electron or hydrogen atom transfer mechanisms, the electron transfer is dominant in the reactions between phenols and ABTS$^{\bullet+}$ within the selected time of reaction (Dong et al., 2015). On the other hand, the reaction between DPPH radical and extracted phenolics is highly dependent on the reaction solvent and stereoselectivity of bulky DPPH radical and both mechanisms could take place in the observed reaction time (Zhang et al., 2018). The IC_{50} value of ABTS$^{\bullet+}$ radical cation obtained for the Trolox was found to be 0.2 mg/ml.

The reducing power of wheat bran and fermented wheat bran extracts was assessed using the FRAP method. It is noticeable that the results obtained by this method are in accordance with the results obtained by DPPH and ABTS methods. The ferric reducing power of wheat bran was 53 nmol TE/g and it reached thevalue of 580 nmol TE/g after 8 days of fermentation. Afterward, this value had a small decrease on the 11th day.

3.4. Gluten content and proteolytic activity

Recently wheat bran has attracted more attention as an ingredient in not just feed but also the food industry since it is rich in dietary fiber, minerals, and folic acid (Prückler et al., 2014). Nevertheless, wheat bran contains high amounts of gluten and therefore represents a problem for people with celiac disease (Walter et al., 2014). Results presented in this paper (Fig. 4) show that the after fermentation of wheat bran with isolate *Bacillus* sp. TMF–2 gluten content was reduced for 99% in 7 days of fermentation. Furthermore, reduction in gluten and gliadin content was not observed after 24 h of fermentation and the reason might be autoclaving of wheat bran. Excessive heating can lead to the incorporation of gliadins into glutenin fraction by thiol-disulfide interchange and additionally, it can promote the formation of isopeptide crosslinks in glutenin (Walter et al., 2014). With these newly formed crosslinks, the glutenin structure becomes very compact and more resistant to proteolytic cleavage (Walter et al., 2014). Nevertheless, according to Codex Alimentarius (2015) fermented wheat bran after 168 h of fermentation can be labeled as a gluten-free product. Presented results suggest that strain in use is a producer of protease that can degrade gluten, which is of great interest for the production of gluten-free products. To confirm

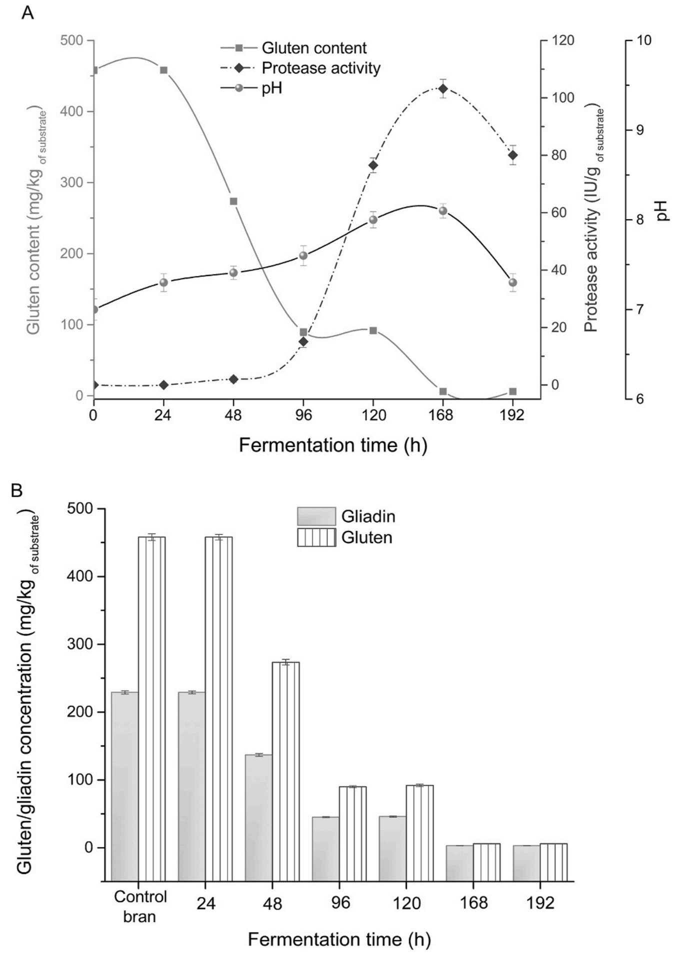

Fig. 4. a) Changes in gluten content, proteolytic activity and pH at different time during fermentation of wheat bran with *Bacillus* sp. TMF–2; b) Gliadin and gluten content of unfermented and fermented wheat bran at different fermentation time. The poined data present mean of three technical repeats ± SD (*n* = 3).

this hypothesis protease activity was measured every day of fermentation and the obtained results are presented in Fig. 4.

As shown in Fig. 4 proteolytic activity is detected on the second day of fermentation, and that is in accordance with the beginning of gluten degradation. As the fermentation proceeds, the protease activity increases reaching the maximum activity of 103 IU/g on the 7th day of fermentation. The highest rate of gluten degradation occurs between the 1st and 4th days of fermentation when the total proteolytic activity was not high, suggesting that during these first days of fermentation this strain produces specific proteses that can degrade gluten. This in accordance with the pH value during the fermentation since pH value increased. The reason for this phenomenon is that during gluten degradation, amino acids are released and the increase in pH is the consequence of their degradation (Ouoba et al., 2003). The drop in pH is in accordance with the increase of amylolytic activity. Namely, when glucose is present in the substrate, *Bacillus* sp. TMF–2 probably ferments them into organic acids and causing a drop in pH (Aguilar et al., 2019). The increase in sugar content is also visible in Fig. 3. Results sugest that further in the fermentation, additional proteases and peptidases are produced during the fermentation. The value of Pearson's coefficient confirmed the existence of strong negative interaction between protease activity and gluten content with a value of −0.80.

4. Conclusions

This paper showed that wheat bran, an abundant low-cost by-product of wheat flour production, could be used as a substrate for the production of the highly valuable hydrolytic enzymes, while the metabolic activity of producing *Bacillus* sp. TMF–2 led to the reduction of anti-nutritional factors in wheat bran, which are otherwise a major limiting factor for its usage as a feed component. The highest titer of protease and amylase activity was recorded on 7th day of fermentation and amounted to 103 and 107 IU/g $_{\text{of substrate,}}$ respectively. Gluten free wheat bran was obtained after 7 days of fermentation.. Additionally, the production strain was shown as a prospective phytase producer, enabling the reduction of phytic acid by ~34% and an increase in inorganic phosphorus up to 15.5 mg/g $_{\text{of substrate}}$ in resulting fermented wheat bran. The production of all hydrolytic enzymes was strongly correlated with the increased content of phenolic compounds in fermented bran along with substantial antioxidant activity established via a reducing power and the free radical scavenging assays. Further, fermented wheat bran possesses greater free radical scavenging and reducing potential in comparison with unfermented i.e. raw bran. The results presented in this study suggest that solid state ferementation of wheat bran via *Bacillus* sp. TMF–2 could be lucrative process since variety of hydrolytic enzymes are being produced as well as wheat bran of superior nutritional quality.

CRediT authorship contribution statement

Sonja Jakovetić Tanasković: Conceptualization, Methodology, Software, Data curation, Writing - original draft, Supervision. **Nataša Šekuljica:** Resources, Visualization, Formal analysis, Data curation, Validation. **Jelena Jovanović:** Methodology, Investigation, Formal analysis, Data curation. **Ivana Gazikalović:** Investigation, Software. **Sanja Grbavčić:** Methodology, Validation. **Nikola Đorđević:** Methodology, Investigation. **Maja Vukašinović Sekulić:** Resources. **Jian Hao:** Funding acquisition. **Nevena Luković:** Supervision, Writing - review & editing. **Zorica Knežević-Jugović:** Conceptualization, Project administration, Supervision, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they not have conflicts of interest with respect to the work described in this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcs.2020.103159.

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