Increasing the Protein Content of Rapeseed Meal by Enzymatic Hydrolysis of Carbohydrates

Ivo M. Rodrigues,[a] M. Graça V. S. Carvalho,[b,*] and Jorge M. S. Rocha [b]

Enzymatic hydrolysis of rapeseed meal carbohydrates was performed to increase the protein content. Rapeseed meal was first screened and further defatted. Only particles between 0.250 and 0.707 mm in size were used, and the optimum solid/liquid ratio was found to be 10% (w/v). Commercially available carbohydrase enzyme mixtures, Viscozyme® L, Pectinex® Ultra SP-L, and Celluclast® 1.5 L, were used in conditions that minimized protein solubilization from the solid phase. The highest results were achieved with Viscozyme® L, with a carbohydrate extraction yield of 80% (as determined by reducing sugars content expressed as glucose equivalent units) after 24 h of reaction at 45 ºC, pH 3.5, and an enzyme dosage equivalent to 96 fungal beta-glucanase (FBG) added to 5 g of defatted rapeseed meal. The simultaneous use of the different carbohydrases (Viscozyme®, Pectinex®, and Celluclast®) did not improve the carbohydrate hydrolysis when compared to the use of Viscozyme® L alone. Viscozyme® L treatment increased the protein content in the rapeseed meal from 41 to 68%, which allowed the solid to be classified as a protein concentrate.

Keywords: Rapeseed meal; Protein concentrate; Carbohydrate hydrolysis

*Contact information: a: Department of Food Science and Technology, College of Agriculture, Polytechnic Institute of Coimbra, Bencanta, 3040-316 Coimbra, Portugal; b: CIEPQPF, Dep. of Chemical Engineering, Faculty of Sciences and Technology, University of Coimbra, Rua Sílvio Lima, 3030-790 Coimbra, Portugal; * Corresponding author: mgc@eq.uc.pt*

INTRODUCTION

The use of vegetable proteins, particularly from oilseeds and cereals, has increased in recent decades. Vegetable proteins from these plants have been used as alternatives to animal proteins in human nutrition, as functional agents and bioactive compounds, not only in food, but also in cosmetics and pharmaceuticals (Živanović *et al*. 2011). Vegetable proteins are usually present in the form of protein concentrate or protein isolate, although their use is sometimes limited by their characteristics, such as their solubility (Moure *et al.* 2006).

In recent years, rapeseed consumption has greatly increased to meet biodiesel production needs, leading to a large amount of low-economic value meal with a high potential for valorization (Rodrigues *et al.* 2012a). In Portugal, the imports of rapeseed increased from 7,906 tons in 2006 to 292,827 tons in 2010 (FAO 2013). Rapeseed meal is composed of *c.a.* 40% (dry basis) proteins that comprise a balance of various amino acids, which are required in the human diet; this balance of various amino acids confers a high biological value to rapeseed-derived proteins, even higher than other vegetable sources, such as soybean and wheat (Ghodsvali *et al.* 2005; Salunkhe *et al.* 1992). However, when compared to soybean meal, rapeseed meal has a lower protein content and a lower digestibility, due to the high levels of fiber and the presence of

glucosinolates, phytates, and erucic acid (Aherne *et al*. 1976; Badawy *et al*. 1994; Brand *et al*. 2007; Fenwick *et al*. 1983; Liener 1994; Tripathi and Mishra 2007). For instance, glucosinolate degradation products interfere with thyroid function and affect vital organs (Bell 1984; Pirie and Swaninathan 1975; Tripathi and Mishra 2007). Additionally, when compared with other vegetable proteins, rapeseed meal has a lower proportion of carbohydrates, which are mostly amyloid compounds, cellulose, pectins, and arabinogalactan. Cellulose is mostly found in the hull, together with hemicellulose and lignin. The main sugar component is sucrose, followed by smaller amounts of its oligosaccharide derivatives, raffinose, and stachyose. Some digestive problems leading to flatulence in both humans and animals are linked to these last two compounds (Shahidi 1990). For these reasons, rapeseed meal has only been used as a feed for ruminants, which can easily digest these compounds due to their complex digestive system, and its commercial value is significantly low: in Portugal, it is *c.a.* 75% that of soybean meal value (IACA 2013).

The protein content in the solid product determines its classification. It is called protein concentrate when the protein content reaches 65%, and is called protein isolate when the protein level reaches 90% (Kirk-Othmer 1997; Moure *et al*. 2006). The protein fraction of rapeseed meal can be valorized into protein concentrates or protein isolates by using techniques capable of reducing or eliminating the carbohydrates and the compounds responsible for lowering the nutritional value of rapeseed meal. This potential protein source can then compete in the market with other sources of protein used for feed and food supplement.

Protein concentrates are usually obtained through techniques that include the physicochemical extraction of the non-protein material in the meal, primarily carbohydrates, thus increasing the protein fraction in the resulting solid. The extraction techniques for non-protein materials encompass the use of alcoholic aqueous solutions (60 to 80%) and acidic conditions (*e.g*. pH 4.5) to minimize the loss of solubilized protein (Kirk-Othmer 1997; Lewis and Gradison 1996). Protein concentrates obtained in this way retain most of the fiber content in the meal, which is favorable in the case of soybean due to its application in cereal mixtures and as a meat substitute. However, the presence of fibers in rapeseed, the content of which is higher than in soybean, contributes to the low digestibility of its concentrate.

Another strategy for obtaining high-protein concentration products from vegetable sources is by using enzymes to extract protein, which yields protein isolates after precipitation. The use of proteases, Protex® 40XL, Protex® P, and Protex® 5L, to catalyze protein extraction increases the extraction yields to 90% for soybean meal and to 50 to 80% for rapeseed meal (Sari *et al*. 2013). On the other hand, carbohydrases are commonly used to improve the extractability of protein with or without the use of proteases. In fact, for this purpose, the carbohydrases Viscozyme® and Celluclast® have been used in the neutral pH extraction of protein from rice bran (Ansharullah *et al*. 1997). Brewers' spent grain, a high-volume co-product from the brewing industry, treated with the combined action of carbohydrases (Depol™ 740 and Econase®) and proteases (Alcalase® and Promod™ 439), led to the solubilization of more than 80% of the total protein, up to 39% of the total carbohydrates, and up to 42% of the total dry matter (Treimo *et al*. 2009). Simultaneous extraction of protein from distiller's grains with food-grade bio-based solvents and enzymatic saccharification of glucan was used as a strategy to produce a high-value animal feed, with the simultaneous production of additional sugars for bioethanol production (Datta *et al*. 2010).

The carbohydrate fraction of crude rapeseed, an oilseed containing 45 to 50% fat, is composed of 10% soluble sugars, 4 to 5% cellulose, 4 to 5% pectins, and 3% hemi-celluloses (dry basis) (Shahidi 1990). After oil extraction the rapeseed meal exhibits a protein content of *ca.* 40%. A strategy to increase the protein content in rapeseed meal is by the removal of non-protein material from the solid, catalyzed by enzymes. The enzymatic hydrolysis of the carbohydrates in the vegetable matter is less harsh than acid hydrolysis, which leaves the protein structure more intact. Carbohydrates can be hydrolyzed into fermentable sugars, and different types of microorganisms can be used during fermentation to produce a large range of value-added products. Bioethanol production *via* alcoholic fermentation of sugars is just an example.

The aim of this work was the increase of the protein content in rapeseed meal by enzymatic hydrolysis of the carbohydrates. The liquid solution, which is potentially rich in carbohydrates, could be further used for fermentation purposes. Taking into account the carbohydrates composition, three commercially available carbohydrase enzyme mixtures were utilized in conditions that kept the rapeseed protein in the solid phase. This strategy has not been tested to valorize this material, and neither has the use of pectinases.

EXPERIMENTAL

Rapeseed Meal Screening and Chemical Composition

The rapeseed meal was collected from a Portuguese biodiesel production company. It was first screened using different mesh screen sieves (Retsch, ASTM type, Germany). The rapeseed meal as a whole and as screened fractions (*i.e.*, < 0.250 mm, 0.250 to 0.707 mm, and > 0.707 mm) were subjected to centesimal composition analysis following the Weende scheme (Lloyd *et al*. 1978). This analytical procedure (Fig. 1) enables the quantification of moisture, ash, crude fat (ether extract), crude protein, crude fiber (cellulose), and nitrogen-free extract (NFE). The latter was determined by difference.

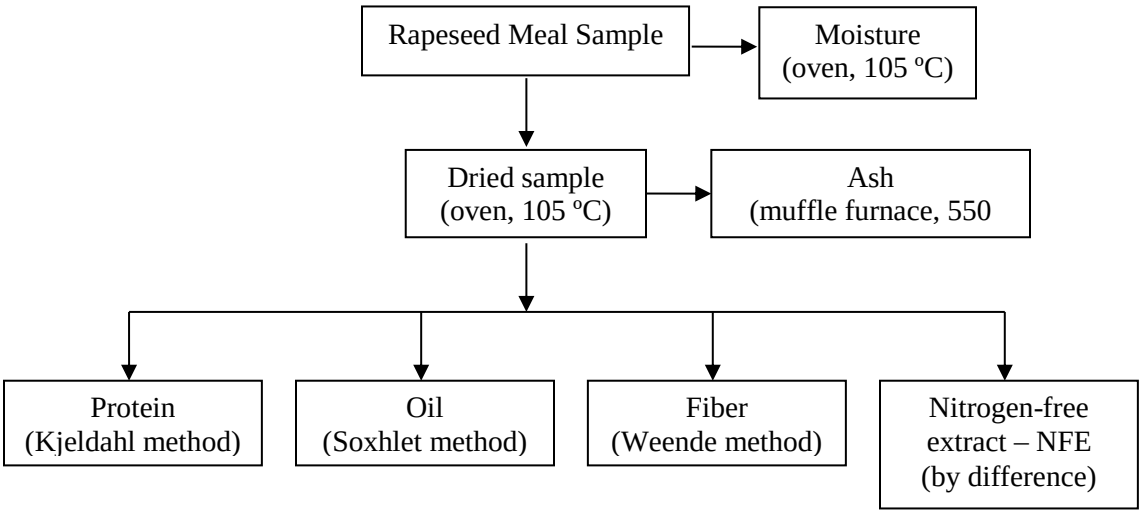


Fig. 1. Weende scheme to evaluate the gross composition of a vegetable sample (Lloyd *et al*. 1978).

For carbohydrate enzymatic hydrolysis, only particles between 0.250 and 0.707 mm were used. Before being subjected to enzymatic hydrolysis, the screened rapeseed meal was first dried at 105 °C (overnight) and subsequently defatted of residual oil by Soxhlet extraction for 5 to 6 h using petroleum ether as the solvent. The extracted rapeseed meal, free of water and oil, is hereafter referred to "treated rapeseed meal". For research purposes, the residual oil was removed to avoid its possible interference in the enzymes performance.

Enzymatic Hydrolysis of Treated Rapeseed Meal

The enzymatic hydrolysis of treated rapeseed meal was performed by carbohydrase treatment. According to the well-known carbohydrate composition as described earlier (Shahidi 1990), aqueous solutions of Viscozyme® L, (from Sigma-Aldrich, Germany), Pectinex® Ultra SP-L, and Celluclast® 1.5 L (from Novozymes, Denmark) were used. Viscozyme® L is a multi-enzyme complex containing a wide range of carbohydrases, including arabinase, cellulase, *β*-glucanase, hemicellulase, and xylanase. Pectinex® Ultra SP-L is an enzyme complex with a high pectolytic activity containing some hemicellulolytic activity. Celluclast® 1.5 L is an enzyme complex that catalyzes the breakdown of cellulose into glucose, cellobiose, and glucose oligomers. Table 1 presents the activity and the recommended reaction conditions suggested by the manufacturers for these enzymatic mixtures.

Table 1. Activity and Optimal Reaction Conditions of the Enzymatic Mixtures Selected for This Work (Data Supplied by Manufacturers)

Commercial enzyme complex (aqueous solutions)	Density (kg/L)	Activity[a]	Recommended pH	Recommended temperature (ºC)	Producer strain	Supplier
Viscozyme® L	1.2	100 FBG/g	3.3 to 5.5	40-50	*Aspergillus* sp.	Sigma-Aldrich
Pectinex® Ultra SP-L	___	26,000 PG/mL	3.5	35	*Aspergillus aculeatus*	Novozymes
Celluclast® 1.5L	1.2	700 EGU/g	4.5 to 6.0	50-60	*Trichoderma reesei*	Novozymes
[a]FBG - Fungal beta-glucanase; PG – Polygalacturonase; EGU - Endo-glucanase units						

The majority of the enzymatic hydrolyses were performed with a solid/liquid (S/L) ratio of 10% (w/v) (*e.g*. 5 g of treated rapeseed meal in 50 mL of water). To investigate the effect of the S/L ratio, additional S/L ratios of 5% and 15% (*e.g*. 2.5 and 7.5 g of treated rapeseed meal in 50 mL of water, respectively) were explored.

The enzymes were added to the suspensions, and the enzymatic hydrolysis was carried out at temperatures between 35 and 55 ºC in an orbital incubator (Stuart, 500 SI, UK) at 200 rpm. The reducing sugars content was measured up to 24 h of reaction. The pH of the reaction mixture was adjusted with 1M H_2SO_4 to a pH of between 3.5 and 4 to avoid protein solubilization but to allow for enzymatic hydrolysis (Ghodsvali *et al*. 2005; Pedroche *et al*. 2004).

Figure 2 shows the methodology used in the enzymatic hydrolysis of the carbohydrate component of treated rapeseed meal samples.

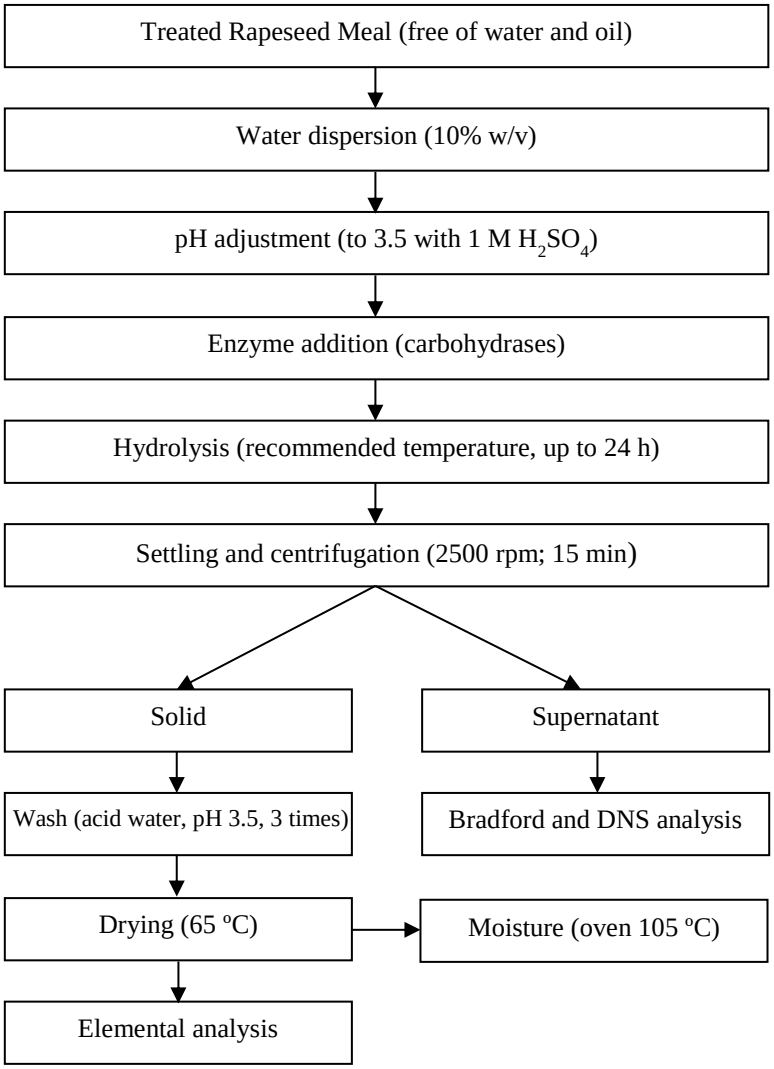


Fig. 2. Strategy for enzymatic hydrolysis of carbohydrates from treated rapeseed meal

After reaction, the mixture was allowed to settle and the liquid extract was centrifuged at 2500 rpm for 15 min (Hettich, Universal model 320, Germany). The supernatant was analyzed for its reducing sugars content by the dinitrosalicylic (DNS) acid method (Miller 1959; Solís *et al*. 2009) and for its protein content by the Bradford method (Bradford 1976; Cao *et al*. 2010; Rodrigues *et al*. 2012b). The solid material was dried overnight in an oven at 65 ºC. In some trials, this material was analyzed by elemental analysis (Fisons Instruments EA 1108 CHNS-O, USA) and by the Kjeldahl method to compare the results obtained for protein quantification from different methodologies.

Determination of Protein Content in Protein Concentrates

The protein content of the solid phase obtained after the enzymatic hydrolysis of rapeseed meal was evaluated indirectly taking into account that: i) the protein content in the initial sample was determined by the Kjeldahl method and confirmed by elemental analysis (% protein = % N * 6.25), ii) the protein loss in liquid extracts during enzymatic hydrolysis of carbohydrates was quantified by the Bradford method, and iii) the content

of reducing sugars present in liquid extracts was analyzed by the DNS method. Equation (1) was used to calculate the protein content in concentrates.

$$\text{Protein in concentrates (\% w/w)} = \frac{\text{Protein in initial sample (g)} - \text{Protein in liquid extract (g)}}{\text{Mass of initial sample (g)} - \text{Reducing sugars in liquid extract (g)} - \text{Protein in liquid extract (g)}} \times 100 \quad (1)$$

The protein content of the concentrates evaluated by the global mass balance (Eq. 1) was confirmed to be reliable because Kjeldahl method and elemental analysis were also used in four random trials and similar results were obtained regardless of the enzyme applied.

Determination of Reducing Sugars Extraction Yields

The extraction yield of reducing sugars was obtained from the reducing sugars content in the liquid extract, reported as the sum of the NFE (nitrogen-free extract) and the fiber contents in the treated rapeseed meal initial sample. Reducing sugar yields were determined by Eq. (2).

$$\text{Reducing Sugar Yield (\% w/w)} = \frac{\text{Liquid extract volume (mL)} \times [\text{Reducing Sugar}] \text{ in extract (mg/mL)}}{\text{Mass of initial sample (g)} \times ([\text{NFE}]+[\text{Fiber}]) \text{ in sample (mg/g)}} \times 100 \quad (2)$$

The reducing sugar content in liquid extracts, which was expressed in glucose equivalents (when Viscozyme® L or Celluclast® 1.5L was used) or in galacturonic acid equivalents (when Pectinex® Ultra SP-L was used alone), was analyzed by the DNS method. The corresponding content was obtained using the following standard curves: [Glucose (mg/mL)] = 2.209 x $O.D._{(540\ nm)}$ + 0.0618, R^2= 0.996 and [Galacturonic Acid (mg/mL)] = 2.6542 x $O.D._{(540\ nm)}$ + 0.0587, R^2= 0.999 where O.D. is the optical density. This information made it possible to follow enzymatic hydrolysis directly and can be used in further studies for fermentation purposes. The reducing sugar content in liquid extracts was not corrected for reducing sugar contribution from the added enzymes solution, as the corresponding amount was negligible (less than 1%).

Statistical Analysis

All experiments were performed in triplicate. Data are expressed as the mean values ± standard deviation (SD) derived from triplicate determinations. The statistical analysis of the data was done by analysis of variance (ANOVA) using the software Minitab® (version 16.2.3 (2012); Minitab Inc., Pennsylvania, USA). Results were considered statistically significant for *p*-values below 0.05.

RESULTS AND DISCUSSION

Rapeseed Meal Screening and Centesimal Composition

The screened mass fractions of rapeseed were 41.7, 50.8, and 7.5% for particle sizes of > 0.707 mm, 0.250 to 0.707 mm, and < 0.250 mm, respectively. The second highest mass fraction corresponded to the largest particles (> 0.707 mm), which was not representative of rapeseed meal core because of the large amount of hulls.

The composition of the overall untreated rapeseed meal, as well as each of the three screened fractions, is presented in Table 2. The NFE content was the highest,

whereas the protein content was the second most abundant component in all fractions; the only exception was for the < 0.250 mm particle size fraction, where the protein content was higher than the NFE content. Particles larger than 0.707 mm had a fiber plus NFE content level *c.a* 19% higher than particles in the faction 0.250 to 0.707 mm; this was attributed to the presence of hulls in the > 0.707 mm fraction. Particles smaller than 0.707 mm had protein, ash, and fiber plus NFE contents consistent with those reported by Pirie and Swaninathan (1975): ranges of 41 to 45%, 7 to 7.5%, and 46 to 49%, respectively (on a dry basis (db)), for different rapeseed species meals with a fat content of about 2%. Particles smaller than 0.250 mm had a protein content 9.5% higher than the 0.250 to 0.707 mm fraction. However, these very small particles make the valorization process much more difficult to scale-up and can lead to filter clogging problems. This was the main reason for adopting the middle size fraction (particle sizes between 0.250 and 0.707 mm) for further studies for enzymatic hydrolysis assays. In this untreated fraction, the protein content is 38.8% (db) and the oil content is 4.1% (db). For enzymatic assays, this residual oil was removed by Soxhlet extraction in the 0.250 to 0.707 mm fraction. Therefore, on an oil-free basis the protein and the fiber plus NFE contents of this treated rapeseed meal was 40.5% and 52.1%, respectively. For each assay, a sample of 5.00 g contained 2.61 g of fiber plus NFE and 2.02 g of protein.

Table 2. Rapeseed Meal Composition According to Particle Size Distribution[a]

	Centesimal composition, % (w/w)			
Component	Overall crude meal	> 0.707 mm	0.250-0.707mm	< 0.250 mm
Moisture	10.9±0.1	11.0±0.2	11.0±0.0	11.2±0.9
Ash (db)	7.1±0.2	6.5±0.3	7.1±0.1	7.7±0.2
Oil (db)	4.1±0.2	3.7±0.5	4.1±0.0	3.9±0.0
Protein (db)	35.3±0.2	30.2±0.4	38.8±0.1	42.5±0.1
Fiber (db)	6.3±0.1	5.8±0.7	7.5±0.4	5.7±0.1
NFE[b] (db)	47.2±0.5	53.8±0.1	42.5±0.4	40.2±0.1

[a]Mean±SD; [b]NFE – nitrogen-free extract; (db) – dry basis

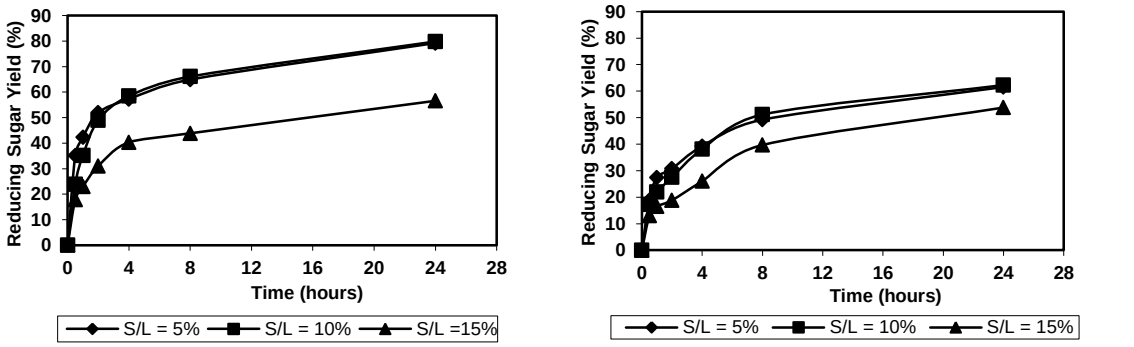
Carbohydrate Hydrolysis of Rapeseed Meal

Effect of solid/liquid ratio

The suspensions of treated rapeseed meal were hydrolyzed using 800 µL of Viscozyme® L (96 FBG units) at 45 ºC for 24 h. The pH was adjusted to 3.5 and did not change significantly during the hydrolysis reaction. The extraction yield of reducing sugars, expressed as glucose equivalents, was determined for S/L ratios of 5, 10, and 15% (w/v) (Fig. 3(a)). Figure 3(b) shows the extraction yields of reducing sugars, expressed as galacturonic acid, over 24 h of carbohydrate hydrolysis catalyzed by Pectinex® Ultra SP-L. These suspensions of treated rapeseed meal were hydrolyzed using 1280 µL of this crude enzyme (33,280 PG units) at 35 ºC and pH 3.5 with S/L ratios of 5, 10, and 15% (w/v). The carbohydrate hydrolysis rate was faster in the first 2 h of reaction, following a hyperbolic profile.

The initial reaction rate and the reducing sugar yield profiles were quite similar for both S/L ratios of 5 and 10% ($p > 0.05$), and this was true for both enzyme mixtures used, Viscozyme® L and Pectinex® Ultra SP-L. Significantly lower values ($p < 0.05$) were obtained for the S/L ratio of 15%, which suggested mass transfer resistance. The reducing sugar yields after 24 h of reaction with Viscozyme® L were 79, 80, and 57% (as

glucose equivalents) for S/L ratios of 5, 10, and 15%, respectively. The corresponding values for Pectinex® Ultra SP-L as galacturonic acid equivalents were 61, 62, and 54%, respectively.



a b

Fig. 3. Effect of solid/liquid ratio (S/L=5, 10, and 15% w/v) on reducing sugars extraction yield profiles over 24 h of hydrolysis at pH 3.5, expressed as: (a) equivalent glucose units, using Viscozyme® L (96 FBG units) at 45 °C; (b) equivalent galacturonic acid units, using Pectinex® Ultra SP-L (33,280 PG units) at 35 °C

For large-scale application, it may be advisable to use a 10% (w/v) S/L ratio to increase the accessibility of enzymes to their substrate, the amount of rapeseed meal treated per volume of hydrolysis solution, and the ease of sample handling. Although the effect of the S/L ratio has not been presented, the same S/L ratio was used in other works with a different objective: to study enzyme-assisted protein extraction from defatted rice bran and oat bran (Tang *et al*. 2002, 2003) and from rapeseed meals and soybean meals (Sari *et al.* 2013).

Effect of enzyme dosage on hydrolysis kinetics and protein concentration

The carbohydrates of treated rapeseed meal were hydrolyzed by Viscozyme® L and Pectinex® Ultra SP-L under the operation conditions suggested by the manufacturer (*i.e.*, pH 3.5 and 45 ºC and pH 3.5 and 35 ºC, respectively) (Table 1). Celluclast® 1.5L was also used under the temperature recommended by the manufacturer (55 ºC – Table 1) but at a lower pH (3.5) to avoid the dissolution of protein. All the suspensions were prepared with a S/L ratio of 10% (w/v), and the reaction was initiated with the addition of 0 to 192 FBG units (fungal beta-glucanase units), 0 to 66,560 PG (polygalacturonase units) and 0 to 672 EGU (endo-glucanase units) of Viscozyme® L, Pectinex® Ultra SP-L and Celluclast® 1.5 L, respectively.

As shown in Fig. 4, the reducing sugars content in the liquid extract increased with time for all the Viscozyme® L dosages, which confirmed continuous hydrolysis activity of the crude enzyme solution during 24 h of reaction. However, the reaction rate was faster in the beginning (within 2 to 8 h), depending on the enzyme dosage. The highest reducing sugar yields were observed for the two highest enzyme dosages, 96 and 192 FBG. Although a slightly higher initial reaction rate was observed for the 192 FBG dosage, the reducing sugar yields after 24 h of hydrolysis reaction were not significantly different ($p > 0.05$), at 80 and 79%, respectively.

Table 3 shows the amount of protein and reducing sugar solubilized in liquid extracts as well as the protein content in the solid phase obtained from the treated

rapeseed meal for each Viscozyme® L dosage. The amount of protein solubilized during carbohydrate hydrolysis was minimal (less than 3% of the total protein in the solid sample) and was similar to the control; this amount of protein included the contribution of the added enzyme.

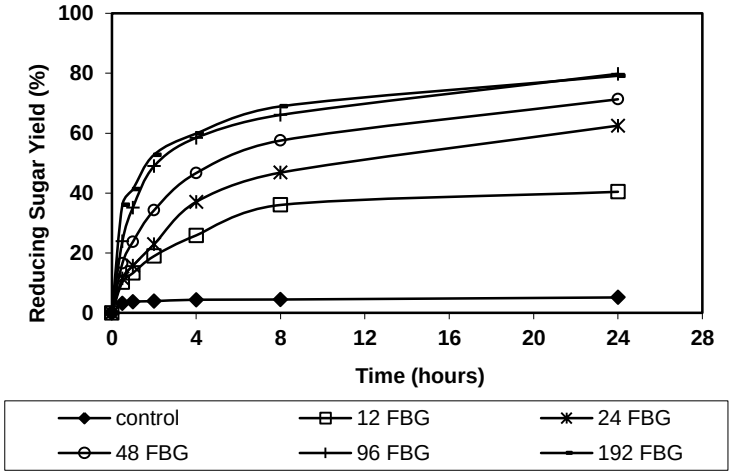


Fig. 4. Reducing sugars extraction yield profiles (expressed as glucose equivalents) for 24 h of hydrolysis with different dosages of Viscozyme® L (FBG units). Solid/liquid ratio of 10% (w/v), pH 3.5, temperature 45 ºC

Protein levels above 60% (w/w) in the solid after 24 h of hydrolysis were achieved with > 24 FBG units of Viscozyme® L. The information presented in Table 3 was also replicated for 8 h of enzymatic hydrolysis, and a protein content of 61.1% was obtained in the solid using a dosage of 96 FBG units. Rapeseed protein concentrates with higher protein content are still obtainable (*c.a* 70%) if higher amounts of Viscozyme® L are used. However, the overall process economy must be considered.

Table 3. Protein Content in Solid Phase after 24 Hours of Hydrolysis with Viscozyme® L at 45 °C, pH 3.5, S/L Ratio 10% (w/v), as a Function of Enzyme Dosage

Enzyme dosage (FBG units)	Treated rapeseed meal sample (g)	Protein in sample (g)[a]	Protein in liquid extract (g)[a]	Reducing sugar in liquid extract (g)[a]	Protein in solid phase (%)[b]
0 (control)	5.00	2.02±0.01	0.058±0.009	0.134±0.007	40.8±0.3
12			0.061±0.005	1.05±0.01	50.4±0.3
24			0.042±0.002	1.63±0.04	59.4±0.8
48			0.049±0.004	1.86±0.03	63.8±0.7
96			0.057±0.004	2.08±0.03	68.6±0.8
192			0.060±0.009	2.06±0.03	68.1±0.9

[a]Mean±SD; [b]determined using Eq. (1)

Regarding Pectinex® SP-L reaction, the reducing sugars content in liquid extracts also increased with time for all enzyme dosages (Fig. 5). The highest reducing sugar yields, 62%, were observed for the two highest enzyme dosages, 33,280 and 66,560 PG

units, after 24 h of hydrolysis reaction. Most of the reducing sugars produced were obtained in the first 8 h of reaction. The highest initial hydrolysis rate and the highest sugar yield at 4 h of reaction were achieved with 66,560 PG units.

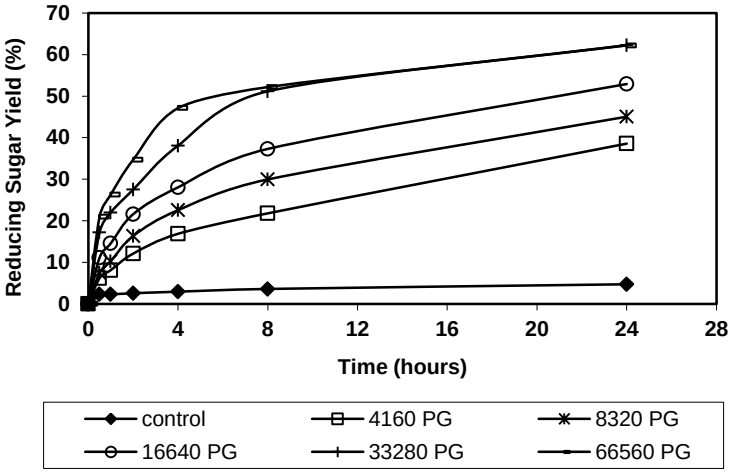


Fig. 5. Reducing sugars extraction yield profiles (expressed as galacturonic acid equivalents) for 24 h of hydrolysis with different dosages of Pectinex® Ultra SP-L (PG units). Solid/liquid ratio of 10% (w/v), pH 3.5, temperature 35 ºC

The amount of protein solubilized in the liquid extract during the carbohydrate hydrolysis with Pectinex® Ultra SP-L was less than 2.5% of the total protein in the solid sample, including the amount of added enzyme – Table 4. A protein content above 50% (w/w) was obtained in the solid after 24 h of hydrolysis with a dosage above 4,160 PG. This mixture of enzymes can also be used to enrich the protein content in rapeseed meals, although the value of 65% (w/w), which is commonly used as a reference target for protein concentrates used as feed (Moure *et al.* 2006), was not achieved.

Table 4. Protein Content in Solid Phase after 24 Hours of Hydrolysis with Pectinex® Ultra SP-L at 35 °C, pH 3.5, S/L Ratio 10% (w/v), as a Function of Enzyme Dosage

Enzyme dosage (PG units)	Treated rapeseed meal sample (g)[a]	Protein in sample (g)[a]	Protein in liquid extract (g)[a]	Reducing sugar in liquid extract (g)[a]	Protein in solid phase (%)[b]
0 (control)	5.00	2.02±0.01	0.031±0.000	0.123±0.003	41.0±0.2
4,160			0.046±0.006	1.01±0.01	50.1±0.3
8,320			0.020±0.001	1.17±0.01	52.5±0.3
16,640			0.029±0.003	1.38±0.01	55.4±0.3
33,280			0.041±0.003	1.62±0.01	59.3±0.4
66,560			0.052±0.002	1.62±0.03	59.1±0.6

[a]Mean±SD; [b]determined using Eq. (1)

In the suspensions prepared with Celluclast® 1.5L, the yield of extracted reducing sugars did not exceed 6.8% (w/w) over 8 h of reaction, even at the highest enzyme dosage tested (672 EGU). These values were much lower than those observed with Viscozyme® L and Pectinex® Ultra SP-L, which demonstrated that this enzyme mixture

had a weak ability to hydrolyze the carbohydrates into monosaccharides. Regarding the rapeseed composition in carbohydrates, which corresponded to 4 to 5% cellulose (db) (Shahidi 1990), and considering the features of the enzyme mixture used, it can be concluded that a very low portion of the cellulose was hydrolyzed into glucose at the end of the 8 h of hydrolysis. As shown in Table 5, the amount of solubilized protein was less than 2.7% of the total protein in the solid sample. However, the protein content in the solid was identical to the control (*c.a.* 40%) for all experiments, as the amount of carbohydrates hydrolyzed by this enzyme mixture was minimal. Therefore, Celluclast® 1.5 L proved to be unsuitable for the production of rapeseed protein concentrates using the strategy suggested in this work.

Table 5. Protein Content in Solid Phase after 8 Hours Hydrolysis with Celluclast® 1.5 L at 55°C, pH 3.5, S/L Ratio 10% (w/v), as a Function of Enzyme Dosage

Enzyme dosage (EGU units)	Treated rapeseed meal sample (g)	Protein in sample (g)[a]	Protein in liquid extract (g)[a]	Reducing Sugar in liquid extract (g)[a]	Protein in solid phase (%)[b]
0 (control)	5.00	2.02±0.01	0.048±0.000	0.099±0.001	40.6±0.2
42			0.054±0.003	0.146±0.006	41.0±0.2
84			0.050±0.002	0.155±0.002	41.1±0.2
168			0.038±0.006	0.159±0.008	41.3±0.3
336			0.037±0.002	0.170±0.010	41.4±0.2
672			0.053±0.004	0.178±0.003	41.2±0.2

[a]Mean±SD; [b]determined using Eq. (1)

It must be highlighted that the protein content in the solid phase calculated by Eq. 1 (mass balance) for the three control trials (without addition of Viscozyme, Pectinex or Celluclast), 40.8, 41.0, and 40.6% respectively, is similar to the value of 40.5% of the treated rapeseed meal. This fact confirms the reliability and consistency of the methodology adopted for protein quantification.

Effect of temperature on the enzymatic hydrolytic activity

Apart from the source of crude enzyme mixtures and dosages, which affect the biocatalytic activity, the reaction temperature is another factor that affects the yield, the kinetics, the viability, and the overall process economy. The three enzyme mixtures were used at three temperatures (35, 40, and 45 ºC) at a fixed S/L ratio of 10% (w/v) and pH 3.5. Each enzyme dosage was selected to be similar to the one that gave good extraction yields of reducing sugars in previous experiments, *i.e.*, 96 FBG units for Viscozyme® L, 16,640 PG units for Pectinex® Ultra SP-L, and 672 EGU units for Celluclast® 1.5L.

Figure 6 shows the profiles of reducing sugars extraction yields, expressed as glucose equivalent units, over 24 h of rapeseed carbohydrate hydrolysis. The effect of higher hydrolysis temperature was shown to be favorable over the tested range, except for Celluclast® 1.5L. This enzyme mixture has a higher optimum temperature (Table 1). The highest results for enzymatic hydrolysis were obtained using Viscozyme® L, which provided an extraction yield of 80% after 24 h of hydrolysis at 45 ºC. Lower yields were obtained at lower temperatures (68% at 40 ºC and 60% at 35 ºC) ($p < 0.05$). The extraction yields obtained with Pectinex® Ultra SP-L after 24 h of hydrolysis were 45% at 35 ºC and 52% at both 40 and 45 ºC, which showed an optimum temperature for this

enzyme that was 5 ºC higher than the manufacturer's recommendations (Table 1). However, the hydrolysis yields with this enzyme were significantly lower than the ones obtained with Viscozyme® L. Celluclast® 1.5L was shown to be the enzyme mixture with the lowest ability to hydrolyze the carbohydrates of the rapeseed meal, providing a reducing sugar extraction yield of 9%, which is too low for practical application.

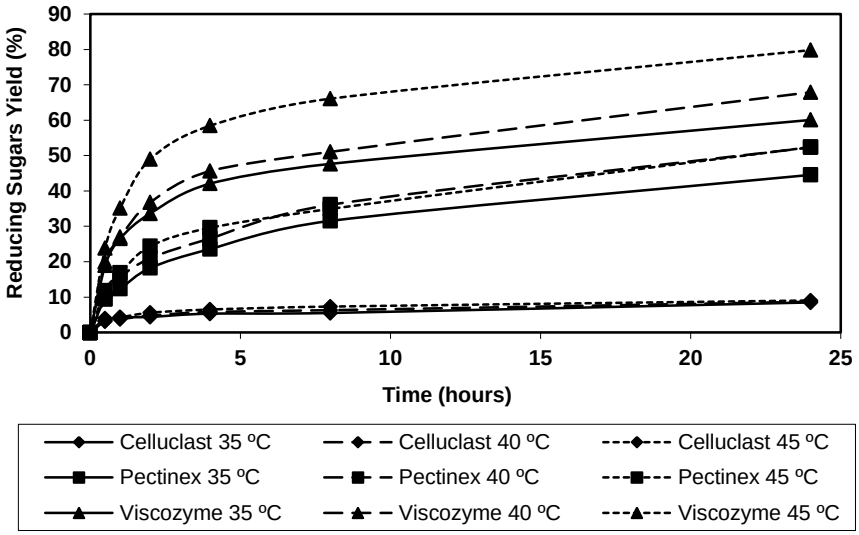


Fig. 6. Reducing sugars extraction yield profiles (all expressed as glucose equivalents) for 24 h of hydrolysis at three temperatures (35, 40, and 45 ºC). Solid/liquid ratio 10% (w/v), pH 3.5. Enzyme dosages: Viscozyme® L - 96 FBG; Pectinex® Ultra SP-L - 16640 PG; Celluclast® 1.5L - 672 EGU

Effect of enzyme mixture on the hydrolytic activity of the rapeseed meal carbohydrates

The commercially available enzyme mixtures used have different carbohydrase compositions. On the other hand, rapeseed meal is a natural compound with different carbohydrates, both in molecular weight and composition. This was the rationale to check the catalytic ability of different enzyme sources. Another strategy that could increase the carbohydrate hydrolysis yield would be to use a mixture of enzymes. Consequently, mixtures of Viscozyme® L and Pectinex® Ultra SP-L (referred to as V+P) and Viscozyme® L, Pectinex® Ultra SP-L, and Celluclast® 1.5L (referred to as V+P+C) were studied at 45 ºC and with identical concentrations used in the single enzymes assays. The temperature of 45 ºC was the optimum value for Viscozyme® L, and this was the optimum temperature found in this work for the activity of Pectinex® Ultra SP-L.

When the performance of V+P and V+P+C mixtures were compared with the results of the single enzyme assays at 45 ºC (Fig. 7), the reducing sugar yield catalyzed by Viscozyme® L alone was still higher (80%) than that obtained with V+P+C and V+P mixtures. In fact, the reducing sugar yields obtained with each mixture were 66 and 62%, respectively, after 24 h of reaction at 45 ºC; this was higher than the yield obtained by Pectinex® Ultra SP-L alone (52%). The amount of protein solubilized in the liquid extract during the enzymatic hydrolysis process did not exceed 3.9% of the total protein in the solid, including the protein content of added enzymes.

The mixture of Viscozyme® L and Celluclast® 1.5L was used by Ansharullah *et al*. (1997) for the treatment of rice bran although with another objective: to improve protein extractability. After 5 h of reaction, at 50 ºC, pH 3.8, the best treatment was the Viscozyme® L alone. Celluclast® 1.5L did not enhance protein extraction, probably

because of the specificity of this fungal enzyme mixture. In a subsequent study, the operation conditions that led to the highest protein concentration in the treated rapeseed meal (free of oil and water) had to be used with crude rapeseed meal, having different amounts of residual oil. A deeper analysis of the concentrates regarding the presence of compounds responsible for the low digestibility, as well as the possible use of the liquid extracts for fermentation purposes, is in progress.

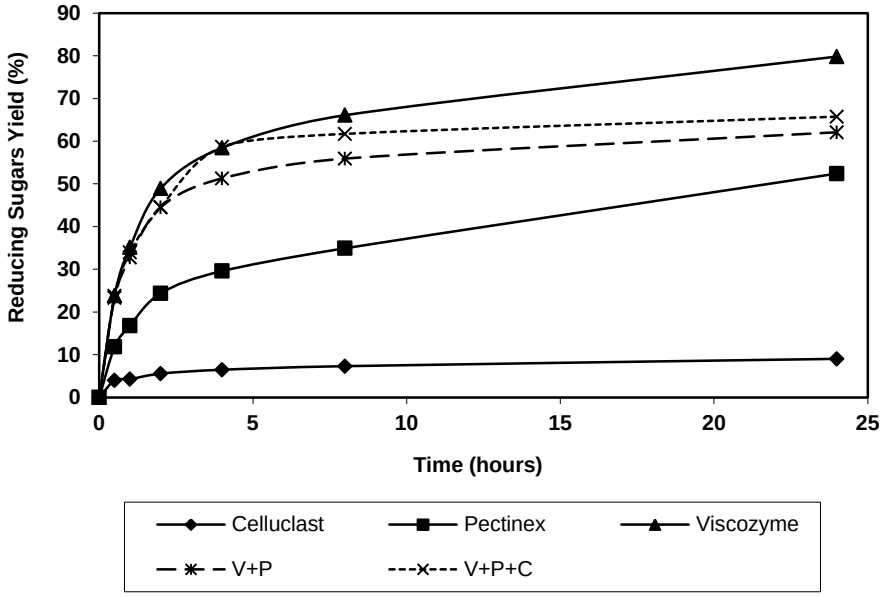


Fig. 7. Reducing sugars extraction yield profiles for 24 h of hydrolysis with optimized dosages of each enzyme mixture tested. Solid/liquid ratio 10% (w/v), pH 3.5, temperature 45 ºC. Dosage of enzymes: Viscozyme® L - 96 FBG units, Pectinex® Ultra SP-L – 16,640 PG units, Celluclast® 1.5L - 672 EGU. V+P means the mixture of Viscozyme® L and Pectinex® Ultra SP-L; V+P+C means the mixture of Viscozyme® L, Pectinex® Ultra SP-L, and Celluclast® 1.5L.

CONCLUSIONS

1. Viscozyme® L was shown to be an efficient biocatalyst for carbohydrate removal in order to increase the protein content in rapeseed meal. An extraction sugars yield of 80%, as glucose equivalent units, was obtained after 24 h of reaction at 45 ºC, pH 3.5, an enzyme dosage equivalent to 96 FBG units, and an S/L ratio of 10 % (w/v). Pectinex® Ultra SP-L allowed an extraction sugars yields lower than 60%. The lowest result was obtained with Celluclast® 1.5 L, with only 9% of extraction sugar yield under the same hydrolysis conditions.
2. A mixture of enzymes (Viscozyme®, Pectinex®, and Celluclast®) did not increase the biocatalytic performance of carbohydrate hydrolysis when compared to the use of Viscozyme® L alone.

3. The S/L ratio of 10% (w/v) was shown to be the adequate suspension concentration of treated rapeseed meal for enzymatic hydrolysis. This S/L ratio optimized the accessibility of enzyme to the substrate with easy sample handling.
4. Viscozyme® L has a strong potential for processing screened and defatted rapeseed meal to protein concentrate. The increase of protein content from 41% to 68% in the resulting solid product allows for its re-classification as protein concentrate.

ACKNOWLEDGMENTS

The authors acknowledge Iberol (Portugal) for providing the rapeseed meal.

REFERENCES CITED

Aherne, F., Bowland, J., Christian, R., and Hardin, R. (1976). "Performance of myocardial and blood seral changes in pigs fed diets containing high or low erucic acid rapeseed oils," *Can. J. Anim. Sci*. 56(2), 275-284.

Ansharullah, J., Hourigan, A., and Chesterman, C. F. (1997). "Application of carbo-hydrases in extracting protein from rice bran," *J. Sci. Food Agric.* 74(2), 141-146.

Badawy, I., Atta, B., and Ahmed, W. (1994). "Biochemical and toxicological studies on the effect of high and low erucic acid rapeseed oil on rats," *Nahrung* 38(4), 402-411.

Bell, J. M. (1984). "Nutrients and toxicants in rapeseed meal: A review," *J. Anim. Sci.* 58(4), 996-1010.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem*. 72(1-2), 248-254.

Brand, T. S., Smith, N., and Hoffman, L. C. (2007). "Anti-nutritional factors in canola produced in the Western and Southern Cape areas of South Africa," *South Africa J. Anim. Sci.* 37(1), 45-50.

Cao, X., Li, C., Wen, H., and Gu, Z. (2010). "Extraction technique and characteristics of soluble protein in germinated brown rice," *Int. J. Food Prop*. 13(4), 810-820.

Datta, S., Bals, B. D., Lin, Y. J., Negri, M. C., Datta, R., Pasieta, L., Ahmad, S. F., Moradia, A., Dale, B. E., and Snyder, S. W. (2010). "An attempt towards simultaneous biobased solvent based extraction of proteins and enzymatic saccharification of cellulosic materials from distiller's grains and solubles," *Bioresour. Technol.* 101(14), 5444-5448.

FAO (2013). FAOSTAT: Trade > Crops and livestock products [WWW Document]. URL http://faostat3.fao.org/home.

Fenwick, G. R., Heaney, R. K., and Mullin, W. J. (1983). "Glucosinolates and their breakdown products in food and food plants," *Crit. Rev. Food Sci. Nutr.* 18(2), 123-201.

Ghodsvali, A., Khodaparast, M. H. H., Vosoughi, M., and Diosady, L. L. (2005). "Preparation of canola protein materials using membrane technology and evaluation of meals functional properties," *Food Res. Int.* 38(2), 223-231.

IACA (2013). Preços Materias Primas [WWW Document]. URL http://www.iaca.pt/index.jsp?page=prices+materials&lang=pt

Kirk-Othmer (1997). "Soybeans and other oilseeds" *Encyclopedia of Chemical Technology*, 4th Ed., Vol. 22, Wiley, New York, pp. 591-619.

Lewis, M. J., and Gradison, A. S. (1996). *Separation Process in the Food and Biotechnology Industries: Principles and Applications*, Woodhead Publ. Ltd., Cambridge.

Liener, I. E. (1994). "Implications of antinutritional components in soybean foods," *Crit. Rev. Food Sci. Nutr.* 34(1), 31-34.

Lloyd, L. E., McDonald, B. E., and Crampton, E. W. (1978). *Fundamentals of Nutrition*, 2nd Ed., W. H. Freeman and Company, San Francisco, CA.

Miller, G. L. (1959). "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Biochem.* 31(3), 426-428.

Moure, A., Sineiro, J., Domínguez, H., and Parajó, J. C. (2006). "Functionality of oilseed protein products: A review," *Food Res. Int.* 39(9), 945-963.

Pedroche, J., Yust, M. M., Megías, C., Lqari, H., Alaiz, M., Girón-Calle, J., Millán, F., and Vioque, J. (2004). "Utilisation of rapeseed protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity," *Grasas y Aceites* 55(4), 354-358.

Pirie, N. W., and Swaninathan, M. S. (1975). *International Biological Programme: Food Protein Sources*, Vol. 4, Cambridge University Press, Cambridge.

Rodrigues, I. M., Coelho, J. F. J., and Carvalho, M. G. V. S. (2012a). "Isolation and valorisation of vegetable proteins from oilseed plants: Methods, limitations and potential," *J. Food Eng*. 109(3), 337-346.

Rodrigues, I. M., Carvalho, M. G. V. S., and Rocha, J. M. S. (2012b). "Valorisation of rapeseed meal: Crucial factors in protein extraction and issues related to protein evaluation method," in: Book of Abstracts of the IAMAW-*1st International Workshop on Valorization of Mediterranean Biowastes and Effluents*, The International Association of Mediterranean Agro-Industrial Wastes, Santarém, Portugal, p. 45.

Salunkhe, D. K., Adsule, R. N., Chavan, J. K., and Kadam, S. S. (1992). *World Oilseeds: Chemistry, Technology and Utilization*, Springer, New York.

Sari, Y. W., Bruins, M. E., and Sanders, J. P. M. (2013). "Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals," *Ind. Crops Prod*. 43, 78-83.

Shahidi, F. (1990). *Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology*, Van Nostrand Reinhold, New York.

Solís, S., Loeza, J., Segura, G., Tello, J., Reyes, N., Lappe, P., Guitérrez, L., Ríos, F., and Huitrón, C. (2009). "Hydrolysis of orange peel by a pectin lyase-overproducing hybrid obtained by protoplast fusion between mutant pectinolytic *Aspergillus flavipes* and *Aspergillus niveus* CH-Y-1043," *Enzyme Microb. Technol.* 44(3), 123-128.

Tang, S., Hettiarachchy, N. S. and Shellhammer, T. H. (2002). "Protein extraction from heat-stabilized defatted rice bran. I. Physical processing and enzyme treatments," *J. Agric. Food Chem.* 50(25), 7444-7448.

Tang, S., Hettiarachchy, N., and Shellhammer, T. H. (2003). "Protein extraction from heat-stabilized defatted rice bran: II. The role of amylase, celluclast, and viscozyme," *J. Food Sci.* 68(2), 471-475.

Treimo, J., Westereng, B., Horn, S. J., Forssell, P., Robertson, J., Faulds, C. B., Waldron, K. W., Buchert, J., and Eijsink, V. G. H. (2009). "Enzymatic solubilization of

brewers' spent grain by combined action of carbohydrases and peptidases," *J. Agric. Food Chem.* 57(8), 3316-3324.

Tripathi, M. K. and Mishra, A. S. (2007). "Glucosinolates in animal nutrition: A review," *Anim. Feed Sci. Technol.* 132(1-2), 1-27.

Živanović, I., Vaštag, Ž., Popović, S., Popović, L., and Peričin, D. (2011). "Hydrolysis of hull-less pumpkin oil cake protein isolate by pepsin," *World Acad. Sci. Eng. Technol.* 51, 53-57.

Article submitted: October 15, 2013; Peer review completed: December 22, 2013; Revised version received and accepted: February 9, 2014; Published: February 18, 2014.