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Two-Stage Countercurrent Enzyme-Assisted Aqueous Extraction Processing of Oil and Protein from Soybeans

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Abstract Enzyme-assisted aqueous extraction processing (EAEP) is an increasingly viable alternative to hexane extraction of soybean oil. Although considered an environmentally friendly technology where edible oil and protein can be simultaneously recovered, this process employs much water and produces a significant amount of protein-rich aqueous effluent (skim). In standard EAEP, highest oil, protein and solids yields are achieved with a single extraction stage using 1:10 solids-to-liquid ratio (extruded flakes/water), 0.5% protease (wt/g extruded flakes), pH 9.0, and 50 °C for 1 h. To reduce the amount of water used, two-stage countercurrent EAEP was evaluated for extracting oil, protein and solids from soybeans using a solids-to-liquid ratio of 1:5–1:6 (extruded flakes/water). Two-stage countercurrent EAEP achieved higher oil, protein and solids extraction yields than using standard EAEP with only one-half the usual amount of water. Oil, protein and solids yields up to 98 and 96%, 92 and 87%, and 80 and 77% were obtained when using two-stage countercurrent EAEP (1:5–1:6) and standard single-stage EAEP (1:10), respectively. Recycling the second skim obtained in two-stage countercurrent EAEP enabled reuse of the enzyme, with or without inactivation, in the first extraction stage producing protein with different degrees of hydrolysis and the same extraction efficiency. Slightly higher oil, protein and solids extraction yields were obtained using unheated skim compared to heated skim. These advances make the two-stage countercurrent EAEP attractive as the front-end of a soybean biorefinery.

Keywords Aqueous extraction · Enzyme · Oil extraction · Countercurrent extraction · Biorefinery

Introduction

Countercurrent extraction with hexane has long been used to extract vegetable oil from seeds; however, due to increasingly restrictive environmental regulations and health concerns, alternatives to oil extraction with organic solvents have been sought [1]. Aqueous extraction processing (AEP) has emerged as an alternative to oil extraction with hexane for different oilseeds [2, 3]. AEP has been considered to be an environmentally clean technology, enabling simultaneous recovery of oil and protein; however, low oil extraction, difficulty de-emulsifying to recover free oil when emulsions are formed, and absence of high-value uses for the resulting aqueous effluent (skim) constitute challenges [2, 4].

In order to overcome the low oil extraction efficiency of AEP caused by incomplete disruption of the cellular structure in the oil-bearing material [5], enzyme treatments [6, 7] and mechanical processes, such as flaking and extruding [8], have been used to improve oil and protein extraction from soybeans. AEP of ground soybeans (full-fat flour) has achieved ~65% extraction of the total oil [9] while enzyme-assisted AEP (EAEP) of flaked and extruded soybeans has achieved oil extraction yields of 93% and 96–97% when using Protex 7L (0.5%) and Protex 6L (0.5 and 1.0%), respectively [10].

The oil and protein extracted during the EAEP of flaked and extruded soybeans are distributed in three fractions

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known as free oil, skim (protein- and sugar-rich aqueous phase) and oil-rich cream emulsion. Two important challenges that need to be overcome to maximize the total oil recovery are cream de-emulsification to produce free oil and recovery of the oil present in the skim fraction. We recently achieved total de-emulsification of the cream fraction generated in single-stage of flaked and extruded soybeans (low solids-to-liquid ratio, 1:10) through the use of enzyme (2.5% of Protex 6L) or chemical (pH 4.5) treatments [10]. Although the oil is considered extracted from the starting material, no methods are yet available to recover the oil present in the skim fraction.

Generally, extraction conditions that favor protein extraction also favor oil extraction [9, 10]. Higher oil extraction is related to solubilizing and/or hydrolyzing protein [2, 9], which is attributed to the breakdown of protein network and oleosin membrane (membrane surrounding lipid bodies composed of lipophilic protein and phosphatides) [11], thereby releasing free oil. Rosenthal et al. [9] reported that protein extraction from soybean flour decreased from 87 to 77% when increasing the solids-to-liquid ratio from 1:25 to 1:6, however, oil extraction was not significantly affected.

Solids-to-liquid ratios around 1:10 have been typically used in EAEP of extruded (flaked/unflaked) soybeans in single-step extraction [8, 10, 12] and this use of large amounts of water for extraction generates high effluent production, known as the skim fraction. The skim fraction obtained in standard single-stage EAEP from flaked and extruded soybeans is a dilute protein effluent with limited uses and opportunities for upgrading and capturing the protein. Depending on the operational conditions used (extraction time, pH, temperature, and type and concentration of enzyme), the skim fraction could contain 12–17% of the oil and 73–87% of the protein present in the starting material (extruded soybeans) [10]. Removing water from AEP protein products, usually by spray-drying, is the most expensive step [4]. Reducing the amount of water used in EAEP and consequently the aqueous effluent volume, without reducing oil and protein extraction yields, is important to making EAEP commercially viable. Based on this need, the use of a multi-stage countercurrent system could counterbalance the loss of the extraction efficiency when reducing the amount of water used, and reduce the cost of separating and/or concentrating the components present in the skim fraction. The key principle of the countercurrent system is contacting the freshest flakes with the richest extraction media, where flakes progress through the process until near-depleted flakes contact fresh extraction media [13].

AEP and EAEP have been shown to produce better quality rapeseed [14], corn germ [15] and soybean [16] oils than conventional hexane extraction. Generally, the oils produced by EAEP have low phosphatide levels (making possible physical refining), low peroxide values, and similar free fatty acid contents in relation to those obtained by conventional processes. Corn germ and soybean oils obtained by EAEP are also more stable against oxidation [15, 16] probably because the oil is exposed to less severe heat in EAEP than in conventional processes.

The objectives of the present study were: (1) to evaluate different solids-to-liquid ratios in single-stage EAEP (hereafter referred to as the standard EAEP) of extruded full-fat soybean flakes; (2) to determine the efficiency of two-stage countercurrent EAEP to increase the solids-to-liquid ratio; and (3) to determine the need for enzymes in the first extraction stage of two-stage countercurrent EAEP.

Materials and Methods

Full-Fat Soybean Flakes

Full-fat soybean flakes were prepared from variety 92M91-N201 soybeans (Pioneer a DuPont Company, Johnston, IA, USA) harvested in 2006. The soybeans were cracked using a corrugated roller mill (model 10X12SGL, Ferrel-Ross, Oklahoma City, OK, USA) and aspirated using a cascade aspirator (Multi-aspirator, Kice, Wichita, KS, USA) to remove hulls, and the meats were conditioned at 60 °C in a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH, USA). The conditioned meats were flaked to approximately 0.25 mm thickness using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA, USA).

Extruding Soybean Flakes

The moisture content of the flakes ($\sim$8%) was increased to 15% by spraying water onto the beans while mixing in a Gilson mixer (model 59016A, St. Joseph, MO, USA). The soybean flakes were extruded at 100 °C barrel temperature and 100 rpm rotational speed with a high-shear geometry screw in a twin-screw extruder (18-mm screw diameter, Micro 18, American Leistritz Extruders, Somerville, NJ, USA). About 80 g of extruded flakes were collected directly into a 1-L beaker containing water. Depending of the process used, additional water was added to achieve different solids-to-liquid ratios. The extruded flakes contained 22.6% oil (as is), 34.5% protein (as is) and 12.2% moisture.

Enzyme Treatments

Endoprotease Protex 6L, obtained from the Genencor Division of Danisco (Rochester, NY, USA), was used in all experiments. Protex 6L is a bacterial alkaline protease derived from a selected strain of *Bacillus licheniformis* and has highest activities at pH 7.0 to 10.0 and 30–70 °C. The enzyme dosage (0.5%) used in extraction was based on the weight of extruded flakes. The dosage and type of enzyme were chosen based on our previous work [10].

Standard EAEP

Evaluating the effects of different solids-to-liquid ratios on oil, protein and solids extraction yields from flaked and extruded soybeans was performed following the standard EAEP flow diagram (Fig. 1). Solids-to-liquid ratios of 1:5, 1:6, 1:8, and 1:10 were evaluated. A higher solids-to-liquid ratio (1:4) was attempted, but the low water level did not allow adequate stirring. The slurry pH was adjusted to 9.0 before adding 0.5% Protex 6L (wt/extruded flakes) and stirred for 1 h at 50 °C. Following extraction, the slurry was centrifuged at 3,000*g*. After removing the insoluble fraction, the liquid phase (skim, cream, and free oil) was placed in a separatory funnel and allowed to settle overnight in a cold room at 4 °C. The liquid phase was fractionated into two fractions: skim and cream + free oil.

Two-Stage Countercurrent EAEP

Two-stage countercurrent EAEP was performed over a 3-day period, with one extrusion each day. The extruded flakes were subjected to two-stage extraction and the skim obtained after the second stage of extraction was recycled to the first extraction stage (incoming fresh flakes) on the following day (Fig. 2). During the first day of extraction, AEP was performed with extruded flakes using 1:6 solids-to-liquid ratio. The slurry pH was maintained at pH 8.0 and stirred for 15 min at 50 °C. The slurry obtained in the first extraction stage was centrifuged at 3,000*g* to remove the insoluble fraction. The liquid phase was separated into skim A and cream A (also containing the free oil) by using a separatory funnel. The insoluble fraction obtained in the first extraction stage (1st insolubles) was then subjected to a second extraction stage. Prior to EAEP, the 1st insolubles were diluted with water to achieve 1:5 solids-to-liquid ratio (when skim B was used on the following extraction day with enzyme inactivation) and 1:6 solids-to-liquid ratio (when skim B was used on the following extraction day without enzyme inactivation). The slurry pH was adjusted to 9.0 before adding 0.5% Protex 6L (wt/extruded flakes) and stirred for 1 h at 50 °C. The different solids-to-liquid ratios used in the second extraction stage were necessary in order to achieve the proper amount of skim B to be used the following extraction day. Depending on whether enzyme was or was not used in the first extraction stage altered the extraction rate of the 1st insolubles and consequently the amount of extractible material. The slurry obtained in the second extraction stage was centrifuged to separate insoluble and liquid fractions. The liquid phase was separated into skim B and cream B, following the procedures described above. Skim B was used in the first extraction stage on the second day in two different ways: (i) without any heat treatment or (ii) heated for 10 min at 85 °C to inactivate the enzyme. The extractions carried out on the second and third days were performed in the same manner as the first day.

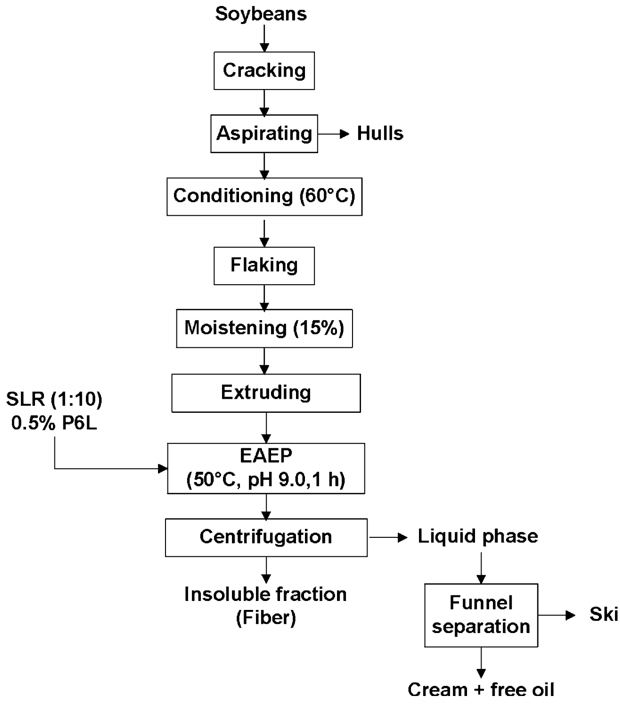

Fig. 1 Process flow diagram for standard single-stage EAEP of flaked and extruded soybeans (*SLR* solids-to-liquid ratio)

Oil, Protein and Solids Recoveries

Analyses of oil, protein and dry matter contents were carried out on the skim, insoluble, and cream fractions as well as the extruded flakes. Total oil contents were determined by using the acid hydrolysis Mojonnier method (AOCS method 922.06), protein contents by using the Kjeldahl method (AACC Standard Method 46-08), and total solids by weighing after drying samples in a vacuum-oven at 110 °C for 3 h (AACC Method 44-40). The extraction yields were expressed as percentages of each component in each fraction relative to the initial amounts in the extruded flakes. Standard EAEP was replicated two times with each replication being a different extrusion. Two-stage countercurrent EAEP was performed in two runs. Each run was for three days, with one extrusion per day and two two-stage extractions per day for each treatment (without or with enzyme inactivation). In order to analyze samples at steady-state, only the duplicate fractions obtained on the third day of each run were analyzed

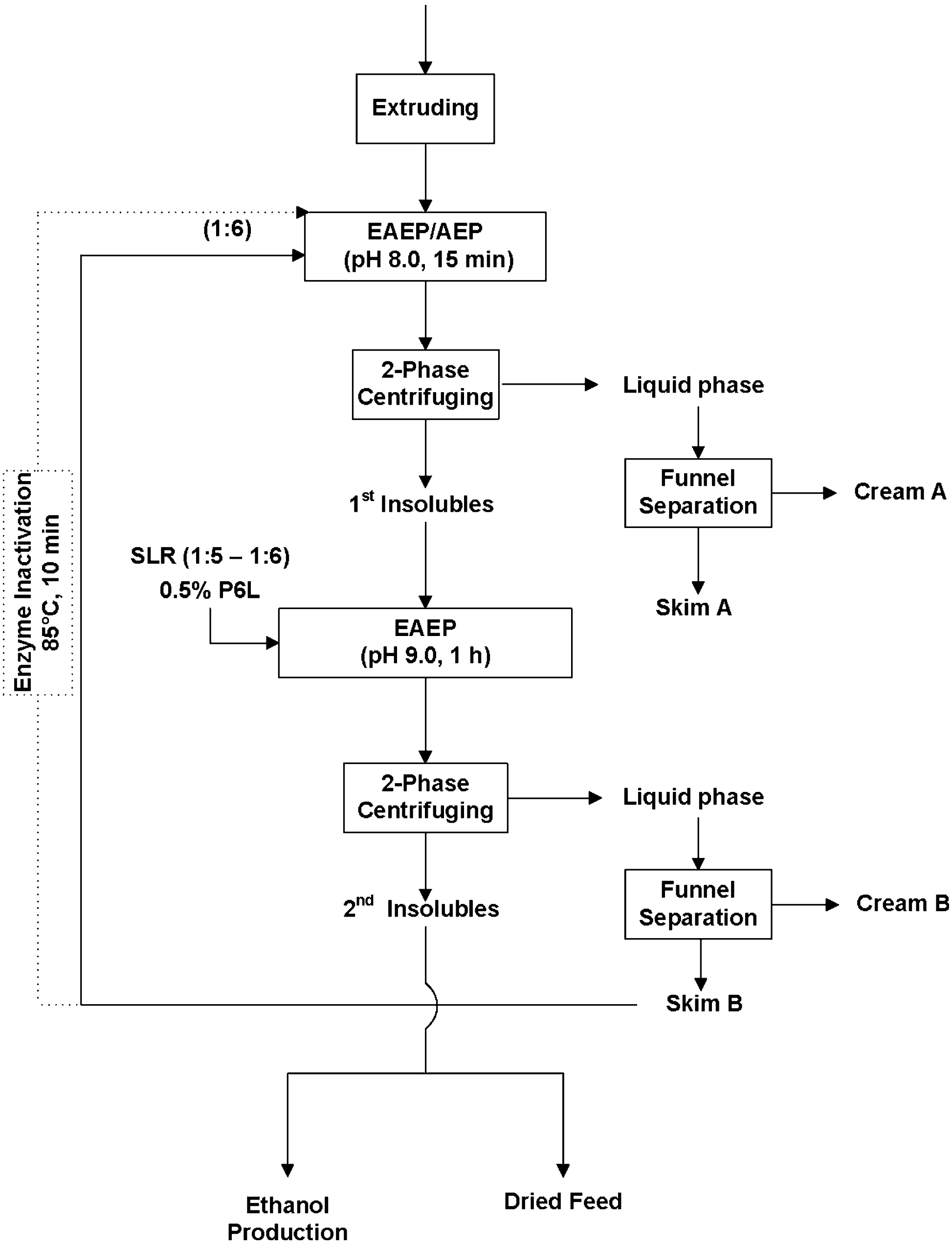

Fig. 2 Process flow diagram for two-stage countercurrent EAEP of flaked and extruded soybeans (*SLR* solids-to-liquid ratio)

for oil, protein and solids contents. All materials collected on days 1 and 2 were discarded (four samples sets were discarded).

Results and Discussion

Effects of Different Solids-to-liquid Ratios on Oil, Protein and Solids Extraction for Standard EAEP

It is well known that the solids-to-liquid ratio influences extraction efficiency [4]. Higher extraction yields have been associated with large quantities of water [2]. Figure 3 shows the effect of different solids-to-liquid ratios on the insolubles composition. Reducing the amount of water used in the standard EAEP increased the oil, protein and solids yields in the insolubles fraction. Increasing the solids-to-liquid ratio from 1:10 to 1:5 increased the residual oil, protein and solids yields in the insolubles fraction from 5 to 10%, 12 to 20% and 23 to 29%, respectively. Although the mechanisms of protein and oil extraction are quite different, high interdependence between oil and protein extractions has been observed [9]. Higher amounts of water favored protein solubilization and to a lesser extent also oil extraction. Higher solids extraction was also favored when using higher amounts of water as was protein extraction.

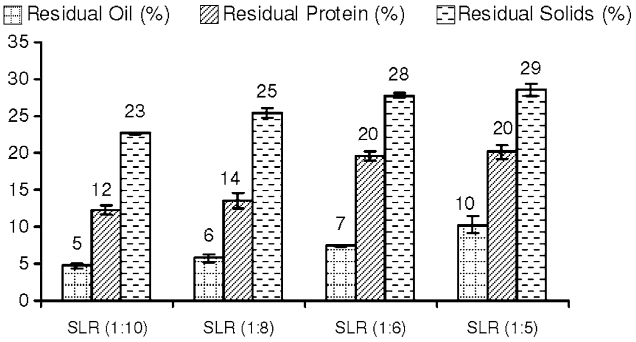

Fig. 3 Effects of different SLRs on the compositions of the insoluble fraction using standard EAEP

Rhee et al. [17] reported decreased oil and protein extraction from raw peanuts from 92.8 to 62.9% and 97 to 70.8%, respectively, when the solids-to liquid ratio (w/v) increased from 1:20 to 1:2. Freitas et al. [12] reported that oil extraction from unflaked extruded soybeans decreased by 2x when the solids-to-liquid ratio increased from 1:10 to 1:3. The results of the present study are in agreement with those reported in the literature for soybeans [9, 10] where generally conditions that favor oil extraction also favor protein extraction. A solids-to-liquid ratio of 1:6 was selected for countercurrent extraction based on the intermediate residual oil and protein values for the insoluble fraction.

Effects of Two-Stage Countercurrent Extraction EAEP on Oil, Protein and Solids Extraction from Flaked and Extruded Soybeans

The effects of using two-stage countercurrent EAEP on oil extraction yields, using heated and unheated skim are shown in Fig. 4a. Recycling unheated skim (containing active enzyme) in two-stage countercurrent EAEP enabled 98% of the oil present in the extruded flakes to be extracted. Extracted oil was distributed as 85% in the cream (83% extracted in the first and 2% extracted in the second extraction), 13% in the skim (6% extracted in the first and 7% extracted in the second extraction), and 2% in the insolubles fraction. The use of heated skim (inactivated enzyme) yielded 95% total oil extraction, of which 79% was distributed in the cream fraction (55% extracted in the first and 24% extracted in the second extraction), 16% in the skim (3% extracted in the first and 13% extracted in the second extraction), and 5% in the insolubles fraction. Using enzymes in both extraction stages (unheated skim) gave slightly higher oil extraction (98%) and produced a skim fraction with lower residual oil (13%) compared to using heated skim. Low oil content in the skim is desirable because oil in the fraction is not recovered as free oil, even though considered extracted from the insoluble fraction. Enzyme (2.5% Protex 6L) and chemical treatment (pH 4.5) were able to totally de-emulsify the cream fraction obtained in EAEP of flaked and extruded soybeans when using 1:10 solids-to-liquid ratio, 0.5% Protex 6L, pH 9.0, 1 h [10]. However, due to the use of higher solids-to-liquid ratio (1:5–1:6) in the two-stage countercurrent EAEP, a cream fraction with different stability properties may result. Investigations regarding the use of enzyme and/or chemical treatments are needed to determine how best to de-emulsify the new cream fraction obtained in the two-stage countercurrent EAEP.

In our previous work [10], 96% oil extraction yield was achieved using standard single-stage EAEP at 1:10 solids-to-liquid ratio (Fig. 4b). The two-stage countercurrent process using unheated skim yielded slightly higher oil extraction (98%) when using 1:6 solids-to-liquid ratio compared to standard EAEP (96% of oil extraction) at 1:10 solids-to-liquid ratio. The two-stage countercurrent process not only compensated for the loss in oil extraction caused by increasing the solids-to-liquid ratio from 1:10 to 1:6 but also improved extraction efficiency by 2 percentage points when using enzyme in both extractions (unheated skim). Higher oil extraction yield obtained by using the two-stage countercurrent process (unheated skim) compared to standard single-stage EAEP can be seen through the higher oil yield in the cream fraction and lower residual oil in the insolubles fraction. The two-stage countercurrent process with unheated skim and the standard single-stage EAEP process yielded skim fractions containing similar oil yields (13–14%).

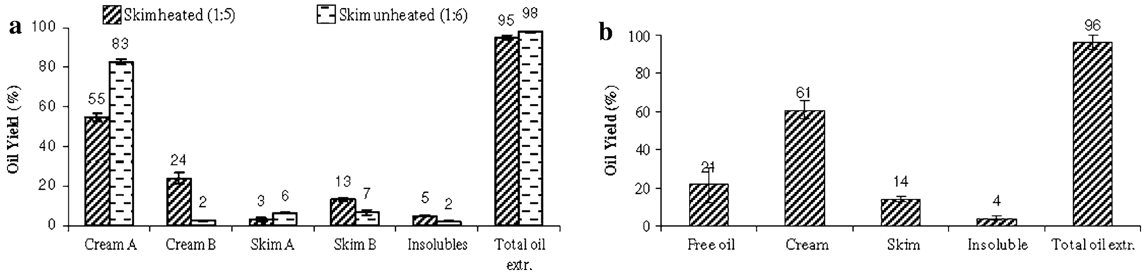

Fig. 4 Oil extraction yields for different EAEP configurations: **a** oil extraction yield in two-stage countercurrent EAEP with heated or unheated skim (1:5–1:6 solids-to-liquid ratio); **b** oil extraction yield in standard single-stage EAEP (1:10 solids-to-liquid ratio; de Moura et al. [10])

Figure 5a shows the effects of recycling the skim, with or without heating, on the protein extraction yield in the two-stage countercurrent EAEP. Using unheated skim gave slightly higher protein extraction yield (92%) compared to using heated skim (89%). The possibility of using heated or unheated skim enabled protein with different degrees of hydrolysis and functionalities to be obtained with almost the same extraction efficiency. Using heated skim in the first extraction stage produced a mixture containing approximately one-third of the protein in its native state (first extraction) and two-thirds partially hydrolyzed (second extraction; MW < 25 kDa [10]).

Two-stage countercurrent EAEP was more effective in extracting protein than standard single-stage EAEP (Fig. 5b) even when using increased solids-to-liquid ratios (1:5–1:6). Contacting the partially extracted insolubles fraction with fresh extraction media in the second stage of extraction compensated for the loss in the protein extraction due to reduced water (Fig. 3) and increased protein extraction yield. Protein extraction yield was increased by 2.0 and 6.0 percentage points when using the two-stage countercurrent process with heated and unheated skim, respectively, compared to the standard single-stage EAEP. In addition to being a source of edible oil, soybeans are considered to be protein crop since 60–70% of the returns in soybean processing are due to meal sales [18]. The protein content in the skim fraction was approximately 60% (dry basis), which can be used to obtain protein isolates or concentrates. In addition to achieving higher protein extraction yield than standard single-stage EAEP, two-stage countercurrent EAEP has another important advantage in that water use is approximately one-half of the water used in standard single-stage EAEP. This water reduction achieves considerable energy savings due to significantly less water evaporation and further separation/concentration of the components present in the skim fraction such as protein and carbohydrates. Stachyose, a flatus oligosaccharide that is associated with reduced nutritional value of soybean protein [19], is present in the skim fraction obtained in single-stage extraction of flaked and extruded soybeans (0.5% of Protex 6L) in levels of 3.8 ± 0.5 mg/mL. Increasing this oligosaccharide concentration by producing lower volume of skim fraction through the use of the two-stage countercurrent would probably reduce costs associated with removing this undesirable carbohydrate.

Figure 6a shows the effects of recycling heated and unheated skims on the solids extraction yields in two-stage countercurrent EAEP. Using unheated skim gave slightly higher solids extraction yield (80%) compared to using heated skim (79%), consistent with oil and protein extraction values presented in Figs. 4 and 5. Comparing the two-stage countercurrent EAEP (Fig. 6a) and the standard single-stage EAEP (Fig. 6a) for solids extraction yields, two-stage

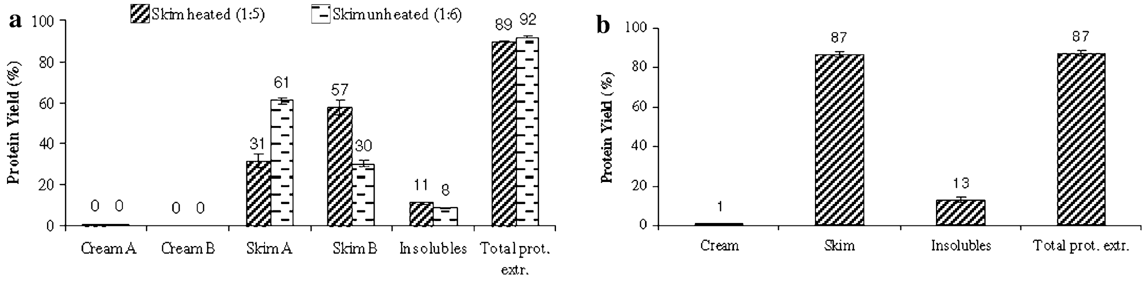

Fig. 5 Protein extraction yields for different EAEP configurations: **a** protein extraction yield in two-stage countercurrent EAEP with heated or unheated skim (1:5–1:6 solids-to-liquid ratio); **b** protein extraction yield in standard single-stage EAEP (1:10 solids-to-liquid ratio; de Moura et al. [10])

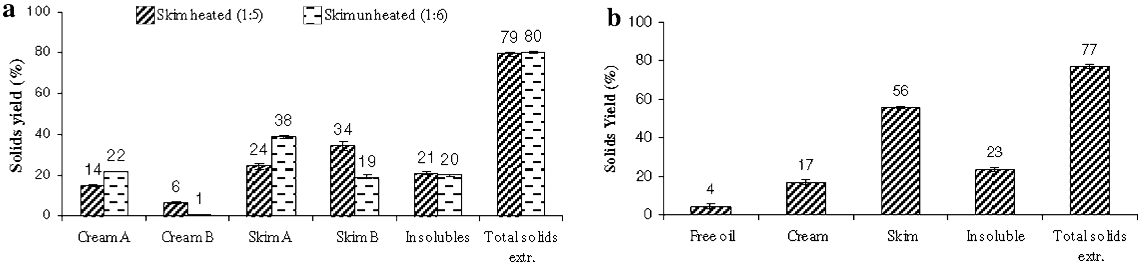

Fig. 6 Solids extraction yields for different EAEP configurations: **a** solids extraction yield in two-stage countercurrent EAEP when using heated or unheated skim (1:5–1:6 solids-to-liquid ratio); **b** solids extraction yield in standard single-stage EAEP (1:10 solids-to-liquid ratio; de Moura et al. [10])

countercurrent EAEP using 1:5–1:6 solids-to-liquid ratio was more effective in solids extraction than standard single-stage EAEP using 1:10 solids-to-liquid ratio. Two-stage countercurrent EAEP using heated and unheated skim improved the solids extraction yield by about 2.5 and 4.0%, respectively, compared to standard single-stage EAEP.

Conclusions

Solids-to-liquid ratio was a very important reaction parameter to oil, protein, and solids extraction in EAEP. Reducing the amount of water used in standard single-stage EAEP from 1:10 to 1:5 increased the residual oil, protein and solids yield in the insolubles fraction, causing lower extraction values. Residual oil, protein, and solids yields increased from 5 to 10%, 12 to 20%, and 23 to 29%, respectively. The use of a two-stage countercurrent system was able to compensate for the loss in the extraction efficiency and even improve extraction efficiency. Higher oil, protein and solids extractions were obtained when using the two-stage countercurrent EAEP with only one-half the water used in the standard single-stage EAEP. The water reduction represents an important energy saving in the recovery of protein and carbohydrates present in the dilute skim fraction. Recycling the second skim (skim B) in two-stage countercurrent EAEP, with or without enzyme inactivation, enables the recovery of protein with different degrees of hydrolysis and functionalities. Slightly better extraction efficiency was observed when using enzyme in both extraction stages.

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