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Enzyme Treatments to Enhance Oil Recovery from Condensed Corn Distillers Solubles

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Abstract The objective of this study was to determine the effect of enzyme hydrolysis of various corn components on oil recovery from condensed corn distillers solubles (CCDS). Hydrolysis with a commercial protease significantly increased oil recovery as the enzyme concentration increased, with the greatest oil recovery being 70% at 10% v/w (dry weight basis) enzyme concentration. Increasing centrifugal force from 8,500 to 12,240×*g* was only slightly effective for the non-enzyme treated samples. Reducing CCDS particle size by grinding with a mortar and pestle increased oil recovery to 83% when an enzyme combination of a commercial cellulase mixture and a protease was used. Particle size reduction of CCDS by high-speed blending resulted in low oil recovery, but the oil recovery was significantly improved after enzyme treatment. Zein-lipid interaction was very strong when tested in a model system, with only 10% of the oil being freed by centrifugation alone. Following enzyme hydrolysis of the zein-oil complex with a protease, oil recovery was increased to 97%. Overall, enzyme hydrolysis and further particle size reduction showed a small, albeit statistically significant, effect in increasing oil recovery from CCDS. These small increases may not justify the use of enzymes or processing modifications to reduce particle size in the ethanol industry, nonetheless, these data may provide a reference or insight to design more effective treatments for oil recovery.

Keywords CCDS · Protein–lipid interactions · Carbohydrate–lipid interactions · Oil recovery · Corn oil

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Introduction

Condensed corn distillers solubles (CCDS), also known as thick stillage produced from drying of the thin stillage, typically contains 35% solids, 14% protein, and 20% oil on a dry weight basis [1]. Comparing to dried distillers grains with solubles (DDGS), CCDS contains more oil, 20 versus 12% w/w [1]. Such oil presents an alternative source for biodiesel production. However, there are challenges in removing the oil from CCDS. There has not been many published systematic research on oil recovery from corn fermentation co-products despite strong economic reasons for doing so.

CCDS is a stabilized heterogeneous matrix because of the shearing and heating during the ethanol production process. Based on our experience and understanding of the matter, we believe that the oil may be present in four forms in the CCDS: (1) as an oil-in-water emulsion stabilized by natural emulsifiers such as proteins, free fatty acids, mono- and di-glycerides, and phospholipids; (2) as minute oil droplets attached to the surface of hydrophobic protein such as zein and also to carbohydrate material or cell wall debris; (3) as intact oil bodies in large endosperm and germ particles having an intact cell structure; and (4) as intact oil bodies released from the broken cellular structure.

The inherent oil compartmentalization in plant seeds consists of lipid bodies (i.e. oleosomes, spherosomes, or oil bodies) with alkaline proteins (oleosins) on the surface [2, 3]. The oil bodies contain oil in the core and are surrounded by a phospholipids monolayer and oleosins interacting with the phospholipids and covering the phospholipids on the outer surface, thereby stabilizing the oil bodies inside the cells [4, 5]. Isolated oil bodies from corn consists of 95% triacylglycerol, 4% diacylglycerol, 0.9% phospholipids, and 1.4% protein (mostly oleosins) [2]. To release the oil

present in this form, oleosomes have to be broken mechanically or chemically.

The cell wall of the corn kernel contains hemicelluloses, celluloses, but no pectin [6]. Therefore, commercial enzyme preparations containing hemicellulases and cellulases may be effective as an enzyme treatment for oil body extraction from CCDS especially from the intact cells. Then a protease treatment should release the oil from the oil bodies. Tzen and Huang [1] hydrolyzed corn oil bodies with trypsin and the oil body membrane broke due to hydrolysis of oleosins. Coalescence then occurred among the oil bodies, so such hydrolysis resulted in oil that could be easily separated from the aqueous medium by gravity. Therefore, if oil in CCDS is stabilized in the oil-in-water emulsion by protein or present in oil bodies released from the broken cell, then a protease treatment should result in oil coalescence and make oil separation by centrifugation feasible.

In the dry-grind ethanol process, the corn is ground by using hammer mill such that there are some relatively large endosperm and germ particles in the corn meal, which can go to the CCDS fraction. Approximately 50% of total corn solid in whole stillage goes to the thin stillage (i.e. CCDS) after decanting [7]. We have also previously found [8, 9] that corn grinding pretreatment can change lipid distribution in liquid stillage and solid cake, however, the oil content in the CCDS highly correlates with its solid content. These CCDS particles most likely contain intact cellular structure, so the use of protease alone may not be effective in releasing the oil. If a protease is used in combination with cellulases and hemicellulases, it may be able to gain access to the proteins encapsulating the oil and release the oil.

In CCDS, the dispersed proteins (which may not be soluble), unhydrolyzed or residual starch, and broken cell walls may interact with the free oil to stabilize it [10]. Proteases can hydrolyze the proteins that interact with the free oil, hemicellulases and cellulases may further break down the fragmented cell wall components which also interact with the oil thus allowing efficient recovery of the oil by centrifugation.

The objectives of the present study were to determine the effect of enzyme hydrolysis of cellular components on corn oil recovery from CCDS, to evaluate the effect of particle size reduction followed by enzyme hydrolysis on oil recovery from CCDS, to understand the interaction of oil with hydrophobic protein zein and protease's effect on such interaction, and to determine how centrifugal force affects oil recovery from CCDS.

Experimental Procedures

CCDS was obtained from LincolnWay Energy, a typical ethanol plant in Nevada, IA. It was stored in a refrigerator at 4 °C prior to analyses. To prevent mold growth of CCDS, sodium azide was added. Different batches of CCDS were obtained throughout the study. For all treatments in each experiment the same batch of material was used. The most important factor, oil content of each batch was determined by the AOAC standard acid hydrolysis method (922.06) [11] and it was used as the base to calculate oil recovery. Pure zein was obtained from Freeman Industries LLC (Tuckahoe, NY, USA), and this industrial-grade zein contained 15.3% nitrogen on a dry weight basis.

Chemicals

Hexanes and ethyl ether were obtained from Fisher Scientific (Fairlawn, NJ, USA), absolute ethanol was obtained from Underwriters Laboratories (Northbrook, IL, USA). All other reagents used were analytical grade.

Enzymes

All the enzymes were obtained from Genencor® International Inc. (Rochester, NY) except for Alcalase 2.4L which was procured from Novozymes (Franklinton, NC, USA). All enzymes were in liquid form and the Genencor® enzymes were commercial preparations. They were stored at 4 °C until use. Six different types of enzymes were used for the study which included acid and alkaline proteases, cellulase, pectinase, amylase, and phospholipases as described below:

1. Acid protease: Protex™ 13FL Genencor® acid fungal protease from *Aspergillus niger* has a declared activity of 1,000 SAPU/g enzyme. One SAPU (Spectrophotometer Acid Protease Unit) is the amount that liberates 1 μmol tyrosine/min from a casein substrate. The optimum pH of the enzyme is 3 and the optimum temperature is 50 °C.
2. Alkaline protease: Alcalase (2.4L type FG), a serine protease extracted from *Bacillus Licheniformis*. The enzyme activity is 2.4 Anson units (AU)/g and has optimum pH and temperature of 9 and 50 °C, respectively. One AU is the amount of enzyme that digests hemoglobin and produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as 1 mequiv. of tyrosine released per min.
3. Cellulase: Multifect® CX GC Genencor® cellulase has cellulase, hemicellulase, xylanase, and glucanase activity. The cellulase is derived from a selected strain of *Trichoderma reesei*. The declared activity is 3,200 U/g, and the suggested optimum activity is at pH 4 and 55 °C.

4. Amylase: Multifect® AA 21L α-amylase with high heat and low pH stability is an endo-amylase from a genetically modified strain of *Bacillus licheniformis*. The recommended temperature at pH 5.5–5.8 is 85–93 °C. The declared activity is 17,400 LU/g. One liquefon unit (LU) is the measure of the digestion time required to produce a color change with iodine solution, indicating the definite stage of dextrinization of starch substrate under specified conditions.
5. Phospholipases: G-ZYME® G999 Lyso-phospholipase is a food grade fungal enzyme produced by fermentation of *Aspergillus niger*. The optimum conditions for the enzyme are pH 4.5 and 60 °C. Activity of the enzyme is 1,000 U/g at pH 4.5 and 60 °C. LysoMax™ microbial phospholipase A2 is a lecithinase produced by microbial fermentation which hydrolyzes the ester bond on the *sn*-2 position of the phospholipids. The optimum conditions for the enzyme are pH 8.5 and 40 °C.
6. Pectinase: Multifect® Pectinase FE is a concentrated liquid pectinase complex from *Aspergillus niger* and contains pectinase, cellulase and hemicellulase activities. The enzyme activity is 145–180 U/g. The optimum temperature of the enzyme is 45 °C and the optimum pH is 3.8.

Compositional Analysis of CCDS

The moisture content was determined by using a vacuum drying oven at 50 °C until a constant moisture content was obtained. Since CCDS caramelizes at higher temperatures, 50 °C was chosen as ideal condition. The combustion method was used for determining the protein content using the VarioMax Carbon Nitrogen analyzer (Elementar Analysensysteme Hanau, Germany) (AOAC method 990.03) [11]. The total oil content was determined by acid hydrolysis (AOAC method 922.06) [11]. Total oil and moisture contents were measured in duplicate for each batch of supplied CCDS during the study.

Enzyme Hydrolysis

Enzyme hydrolysis was carried out under the optimum conditions for each of the enzymes and each treatment was replicated twice. Treatments were done in 250-mL centrifuge bottles using about 40 g of CCDS in a shaker water bath (Model-R-76, New Brunswick Scientific Co. Inc., Edison, NJ) unless otherwise stated. The solids (dry matter) content of the CCDS was adjusted to 30% unless otherwise stated. The enzyme dosage was based on the solids content of the CCDS used in all experiments. The incubation time varied from 3 to 6 h.

Effect of Enzyme and Enzyme Concentration on Oil Recovery

Protex™ 13FL acid protease and Multifect® CX GC cellulase were used to study the effects of increasing enzyme concentrations on oil recovery. The concentration of the enzyme was increased from 0 to 20% (v/w) based on the solids content of the CCDS. Enzyme hydrolysis was carried out at pH 3, and 50 °C for 3 h for the Protex™ 13FL acid protease and at pH 4, and 55 °C for 3 h for the Multifect® CX GC cellulase.

Oil Separation and Quantification After Enzyme Hydrolysis

Following enzyme hydrolysis, oil separation was carried out by centrifugation using a Centra MP4 centrifuge (International Equipment Company, Needham Heights, MA) fitted with a 854 rotor, 20° fixed angle, 7.6 cm radius at 10,000 rpm (8,500×*g*) for 10 min in 50-mL centrifuge tubes. The separated oil was transferred using hexane to wash the top layer for at least five times (10 mL each time). The hexane and oil mixture was transferred to preweighed round-bottom flask. Removal of solvent was done by a rotary evaporation system equipped with a heating bath at 60 °C (Buchi rotavapor R124). Any residual solvent was removed using a vacuum oven at 25 °C. The weight of the oil was then determined gravimetrically.

Effect of Particle Size Reduction of the CCDS on Oil Recovery

We hypothesized that a significant amount of oil is trapped in the solid particles that are present in large amounts in the CCDS. Therefore, we chose to reduce the size of the particles in the sample to test this theory. Three methods were used. The first method was size reduction by sonication, in which CCDS was diluted to 20% solids content. Samples of the thinned CCDS, 45 g, were transferred to 50-mL centrifuge tubes for sonication. Misonix Sonicator® 3000 (Farmingdale, NY) was used which has an operating frequency of 20 kHz and a maximum power output of 600 W. The power used was 390 W and treatment time was 10 min (with ice water bath to keep the temperature at close to ambient). The treated CCDS was transferred to 250-mL centrifuge bottles and about 40 g of CCDS was used. The following enzymes were used for the enzyme hydrolysis after sonication: Alcalase 2.4L alkaline protease and a mixture of Multifect® Pectinase FE and Protex™ 13FL acid protease in equal proportions. The enzyme concentration was 5% (v/w) based on the solids content of the CCDS. For incubation with Alcalase 2.4L alkaline protease

the incubation conditions were pH 9 at 50 °C for 3 h. For incubation with Multifect® Pectinase FE and Protex™ 13FL acid protease, pH 3.5 at 50 °C for 3 h was used. Appropriate controls were used at similar incubation conditions but without the enzyme addition.

The second method of particle size reduction was by grinding. CCDS (35% solids content) was placed in a stack of three sieves with sieve openings of 53 μm (US mesh 270), 106 μm (US mesh 140) and 435 μm (US mesh 40). CCDS particles were partitioned on the sieves with 37% recovered by 53 μm sieve opening, 47% recovered by 106 μm and 16% recovered by 435 μm based on wet weight basis. The larger CCDS particles on the 435 μm sieve opening were subjected to grinding treatment with mortar and pestle and with 11% w/w sea sand added to facilitate particle size reduction. The ground sample was then mixed with the finer particle fractions of CCDS. Enzyme hydrolysis on the remixed CCDS sample was performed using a mixture of Multifect® Pectinase FE and Protex™ 13FL acid protease in equal proportions at pH 3.5, 50 °C for 3 h. The enzyme concentration was 5% (v/w) based on the solids content of CCDS.

The third method was by blending, in which the CCDS (35% solids content) was transferred to a blender (Cuisinart Smart Power 7-speed electronic, East Windsor, NJ) and was "liquefied" (setting) for 30 min at 10 min intervals to maintain the temperature at close to ambient temperature. Following this treatment the CCDS was adjusted to 30% solids content prior to enzyme hydrolysis using a mixture of Multifect® CX GC cellulase, Multifect® AA 21 L α-amylase, and Alcalase 2.4L alkaline protease in equal proportions or Alcalase 2.4L alkaline protease alone. For the combination enzyme treatment, the incubation conditions were pH 4 at 55 °C for 3 h followed by incubation at pH 9, 50 °C for 3 h. For incubation with Alcalase 2.4L alkaline protease the incubation conditions were pH 9, 50 °C for 3 h.

Particle Size Analysis

Particle size analysis of the CCDS was performed using a Malvern Mastersizer® 2000 (Malvern Instruments Ltd., Malvern, UK) with a Hydro 2000MU (wet module) sample dispersion system. The CCDS was added to the dispersion unit (beaker containing deionized water). The stirring speed was set at 1,750 rpm and the sample was added until a laser obscuration between 11 and 14% was achieved. Relative refractive index and absorption values used were 1.33 and 0.001, respectively, according to manufacturer's recommendation. Each sample was analyzed in triplicates. The volume weighted mean ($d_{4,3}$) was used for the particle size analysis.

Interaction of Oil with Hydrophobic Protein (Zein)

Since the protein content in CCDS was about 14% (dry weight basis, by our own analysis), a zein dispersion was made in a manner to roughly mimic the protein content in CCDS with 35% solids content. A 5% w/v zein dispersion in water was made by mixing zein in 70% v/v ethanol and heating to 40 °C to allow maximum dispersion. Ethanol was then removed using a rotary evaporator. When most of the ethanol was removed, zein was diluted back to 5% w/v using deionized water. This procedure created a better aqueous dispersion than directly dispersing zein in water. The zein dispersion was then mixed with 20% w/w oil (based on protein or solids content) in a blender as described previously.

For oil extraction from the zein and oil system, the zein and oil dispersion was subjected to enzyme hydrolysis using Alcalase 2.4L alkaline protease, at 5% v/w enzyme concentration, pH 9, 50 °C for 3 h. The control was performed under the incubation conditions of the enzyme-treated samples but without enzyme addition. A heating treatment was also conducted at 100 °C for 30 min to determine the effect of heat on hydrophobic interactions. Oil separation and quantification were done by centrifugation as previously described.

Effects of Centrifugation Force on Oil Separation

CCDS was subjected to enzyme hydrolysis using a combination of enzymes: Multifect® Pectinase FE and Protex™ 13FL acid protease (1.5% enzyme concentration v/w for each enzyme). Incubation was carried out at pH 3.5, 50 °C for 4 h. For the control set, no enzyme was added but the sample was incubated at the same conditions as the enzyme-treated samples. Following incubation, CCDS was subjected to centrifugation at 8,500, 10,280, and $12{,}240 \times g$. Oil transfer and quantification was performed as previously described.

Electron Microscopy Sample Preparation and Imaging

Original CCDS, CCDS after blending, and CCDS residue obtained after blending and enzyme hydrolysis with a mixture of Alcalase 2.4L alkaline protease, Multifect® CX GC cellulase, and Multifect® AA 21 L α-amylase and removing of the free oil were analyzed by using transmission electron microscopy (TEM). For sample preparation, CCDS was primary fixed using 2% glutaraldehyde (w/v) and 2% formaldehyde (w/v) in 0.1 M cacodylate buffer at pH 7.2, 4 °C for 48 h. The primary fixed samples were rinsed twice in 0.1 M cacodylate buffer at pH 7.2 and then secondary fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. The samples

were then dehydrated using 70% v/v ethanol followed by staining overnight with 2% uranyl acetate in 75% v/v ethanol. The samples were further dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated, and embedded using Spurr's epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA, USA). The resin blocks were polymerized for 48 h at 65 °C. Ultrathin sections of the samples were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN, USA). They were collected onto copper grids followed by capturing of images using JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA) at 200 kV using a Gatan Ultrascan 1000 digital camera (Gatan Inc., Warrendale, PA, USA).

Statistical Analysis

Statistical analysis, i.e. the Analysis of Variance (ANOVA), to determine significant difference among the different treatments was performed using SAS 9.1 (Cary, NC, USA). Least Significant Differences (LSD) were calculated at $P = 0.05$. All treatments were carried out in duplicates and results are shown as the means of two replicates ± standard deviation (SD).

Results and Discussion

The composition of CCDS from three different batches used in this study ranged from 18 to 21% for total lipids, 14 to 19% for protein, and 66 to 68% for moisture content (Table 1). Oil content and moisture level were the most important parameters to consider for this study. Oil recovery was calculated based on the oil content determined by acid hydrolysis for the specific batch of CCDS used. Our experimental results were derived from different CCDS batches, and since large batch-to-batch variations do exist, such variation may have contributed to some differences among similar treatments applied to materials from different batches. More evidence of the heterogeneity of the CCDS is that during cold room storage of CCDS, we observed different degree of destabilization of the CCDS emulsions, as shown by the appearance of a free oil layer during prolonged storage. It is worth noting that these differences made the comparison across different experiments inaccurate, so each experiment should be treated as a stand-alone trial and each experiment was only designed to answer specific questions. Additional research is needed to understand the causes for these product variations.

Table 1 Oil, protein, and moisture contents (%) of the three batches of CCDS used

Composition	Batch	Average % ± SD
Oil (dry basis)	1	17.9[a]
	2	19.4 ± 0.1
	3	21.4 ± 0.6
Protein (dry basis)	1	14.1 ± 0.1
	2	18.7 ± 0.1
	3	ND
Moisture	1	65.9 ± 0.1
	2	68.4 ± 0.1
	3	68.3 ± 0.2

CCDS condensed corn distillers soluble, *ND* not determined

[a] Analyzed by Eurofins Scientific Inc., Des Moines, IA, by acid hydrolysis method

Effects of Enzyme and Enzyme Concentration on Oil Recovery

The oil in CCDS may be present as oil attached to hydrophobic surfaces of protein and polysaccharides, an oil-in-water emulsion stabilized by protein and polar lipids and oil in the oil bodies in intact cells or in oil bodies of the free form. The effects of protease and cellulase on oil release are shown in Table 2. When using Protex™ 13FL at 1% enzyme concentration, oil recovery was increased, from 65 to 68%. At high enzyme concentration (20%), the oil recovery was slightly increased to 70%, however, the increase was not significant above 10% enzyme. The acid protease gave 5% higher oil recovery than the no-enzyme treatment, suggesting that there was hydrolysis of proteins on the free oil bodies subsequently leading to release of oil, and/or destabilization of the oil-in-water emulsion stabilized by protein. The remaining 30% oil that could not be recovered may be trapped by polysaccharides or present in

Table 2 Effects of enzyme and enzyme concentration oil recovery from CCDS

% Enzyme (v/w) dry weight basis	Protex™ 13 FL acid protease % Oil recovery ± SD	Multifect® CX GC cellulase
0	64.9 ± 0.3d	60.1 ± 1.9abc
1	68.4 ± 0.4c	62.1 ± 0.4abc
5	69.3 ± 0.5cb	64.0 ± 0.3a
10	70.3 ± 0.7ab	63.2 ± 1.3ab
15	70.1 ± 0.5ab	57.9 ± 1.9c
20	70.5 ± 0.2a	59.1 ± 3.9bc

Means followed by different letters in the same column indicate a significant difference ($P < 0.05$)

the oil bodies of the large endosperm and germ pieces with intact cells.

The cellulase mixture, Multifect® CX GC did not significantly increase oil recovery at 1% enzyme concentration compared to no enzyme treatment, 62 versus 60%. Oil recovery increased slightly at 5% enzyme concentration to 64%. Further increase of enzyme up to 20% did not result in higher oil recoveries. Although cellulases are effective in breaking down the cell wall polysaccharides and should facilitate oil body release [10], oil may not have been freed without protease treatment. This cellulase mixture also breaks cellulosic cell debris that may trap fine oil droplets. The slight and insignificant increase of oil recovery may indicate that the percentage of oil present in such form may be low or the freed fine oil droplets could not float due to the viscous nature of the material. The control samples (no-enzyme treatments) for the acid protease and cellulase treatments gave different oil recoveries, this can be attributed to the differences in the incubation pHs, pH 3 versus pH 4 with pH 3 giving higher oil recovery.

The enzyme dosage used in this experiment was impractically high because we wanted to examine the maximal potential for oil recovery. The slight reduction in oil recovery with high concentration of cellulase treatment may have been partially due to the additional protein and its oil stabilization effect as an emulsifier.

During dry-grind ethanol production, the corn is ground, starch hydrolyzed, and sugar fermented, releasing much of the oil and oil bodies from the corn [10]. The released free oil can become emulsified in the aqueous system. The dispersed hydrophobic protein can stabilize the oil in the oil-in-water emulsion. Proteases hydrolyze the proteins and destabilize the oil-in-water emulsion, releasing free oil that can be separated and recovered. Oil bodies released into the aqueous medium can only release free oil when mechanically disrupted or enzymes are used to hydrolyze the protein and phospholipid layer of the oil bodies, which protect and maintain the integrity of these particles [12]. Oleosins, the oil body proteins, were susceptible to hydrolysis by trypsin as evidenced by the production of smaller polypeptides and coalescence of maize oil bodies [2].

The cell wall of a corn kernel is comprised of hemicellulose, cellulose but no pectin [6], therefore it was reasonable to use a cellulase with hemicellulase activity for oil extraction. Cellulases are believed to be effective in breaking the cell wall and facilitating oil body release [10]. However, Moreau et al. [13] observed that increasing levels of three cellulases from *Trichoderma reesei* in the hydrolysis of corn germ cell wall components resulted in no obvious increase in oil recovery, suggesting that it may be necessary to use cellulases in combination with proteases to increase oil recovery significantly. In aqueous oil extraction practice, enzymes have been used to increase oil yield by breaking the cell wall and membranes and by hydrolyzing the emulsifying proteins [13]. Various enzyme-assisted aqueous oil extraction processes have been investigated for canola seeds [14], soybeans [15, 16], corn germ [13], and oleosomes from soybeans [17]. Proteases alone or in combination with cellulases significantly improved oil recoveries from flaked soybeans [18]. In our further experiments, combinations of enzymes were used.

Effect of Phospholipases on Oil Recovery

The oil recovery after hydrolyzing with phospholipases was also examined. G-ZYME® G999 Lyso-phospholipase hydrolyzes the ester bond on *sn*-1 position of the 1-acyl-glycerol phosphatide but requires that the fatty acid at the *sn*-2 position be absent. LysoMax™ microbial phospholipase A2 is a lecithinase that hydrolyzes the ester bond at the *sn*-2 position of the phospholipids. The use of these two phospholipases together did not significantly increase oil recovery when compared with no-enzyme treatment, 76.4 versus 76.2%. The phospholipases were expected to hydrolyze the phospholipid half membrane of oil bodies and also the phospholipid-stabilized emulsion. Tzen and Huang [2] also reported that hydrolysis with phospholipase A2 and phospholipase C did not result in hydrolysis of the phospholipids present on the surface of the oil bodies. Huang [4] suggested that oleosins form a mushroom-like covering on oil bodies, making phospholipases inaccessible to the phospholipids due to steric hindrance. Such an inaccessibility factor probably contributed to no increase in oil recovery in our experiment. Phospholipid-stabilized oil-in-water emulsion may also contain a protective protein layer.

Effect of Particle Size Reduction and Enzyme Hydrolysis on Oil Recovery

The efficiency of enzyme hydrolysis is expected to depend on the size of particles and cell distortion [10]. During solvent extraction of oil from oilseeds the seed is cracked and flaked to break and rupture the cells for oil extraction [19]. The critical step in aqueous oil extraction processes is grinding or extrusion because it determines oil yield [10]. Particle size reduction enhances the enzyme diffusion rates so that the enzymes can easily act on the substrates [10].

Three treatments were used to break the CCDS solids into finer particles in order to increase the surface area for enzyme hydrolysis to improve oil recovery. The changes in distribution of the particle size after sonication, blending and grinding by mortar and pestle are shown in Fig. 1. The original CCDS (control) had a peak particle size of 20.0 μm, the sonicated sample had a 6.6 μm peak size, and

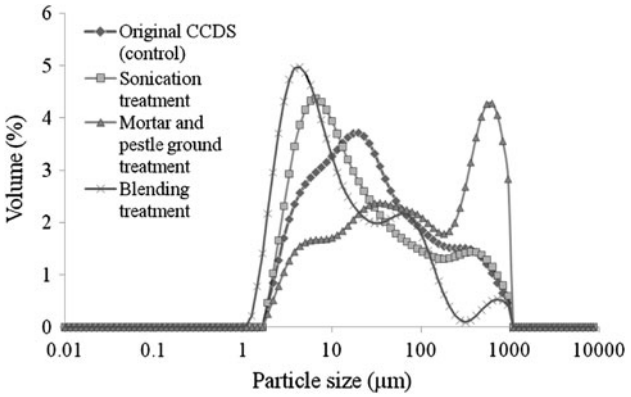

Fig. 1 Particle size distribution profile for CCDS samples treated by sonication, blending, and grinding

the blended sample had a 4.4 μm peak size. The sample prepared by mortar and pestle grinding contained sand, so the CCDS particle size could not be accurately measured. It is possible that by grinding the particle size did not decrease, however, the intense action may have distorted the cells greatly. Therefore, the methods used for particle size reduction seemed effective as indicated by not only the peak particle sizes but also the distribution profiles (except for grinding) as shown in Fig. 1.

The high shear forces of sonication and blending created stable emulsions such that very little oil was separated after enzyme treatment and centrifugation. Oil recovery after sonication and enzyme treatment could not be quantified since there was no free oil formed, but only a cream layer after centrifugation.

Oil recovery after blending the CCDS is shown in Table 3. Three different enzymes were used together for the hydrolysis treatment: Multifect® CX GC cellulase, Multifect® AA 21L α-amylase and Alcalase 2.4L alkaline protease. Previously when cellulase was used alone, it did not significantly increase oil recovery, so it was used in combination with a protease and an amylase for this experiment. Alkaline protease was chosen because it is a more pure enzyme compared to other commercial enzyme preparation, and it was expected to be more effective. The α-amylase was used because even after fermentation of the corn, residual starch may remain due to poor hydrolysis and incomplete fermentation [20] and such residual starch may have formed hydrophobic interactions with the lipids.

In general, blending CCDS resulted in lower oil recoveries even after enzyme hydrolysis compared to samples without blending. Stable emulsion was produced due to severe mixing. The percentage oil recovery after hydrolyzing with a combination of enzyme was 65% (without blending) and 49% (with blending). Slightly higher percentage oil recovery was obtained after hydrolyzing with alkaline protease alone, 68% (without blending) and 59% (with blending). The no-enzyme treatments (controls) gave slightly lower oil recoveries for both the blended CCDS and the not-blended CCDS. The two no-enzyme treatments (controls) were incubated under different conditions with one control being incubated at pH 9, and 50 °C for 3 h while the other control was incubated at pH 4, and 55 °C for 3 h followed by further incubation at pH 9, and 50 °C for 3 h, because the reaction conditions of the enzyme treatments were different. For the no-enzyme and blending treatments at pH 4 and 9, oil recovery was 21% and when combination of cellulase, α-amylase and protease was used, oil recovery increased to 49%, an increase of 128% in oil recovery by enzyme and blending. The no-enzyme and blending treatment at pH 9 achieved 41% oil recovery and when alkaline protease was used, the oil recovery increased to 59%, an increase of 45%. These data indicate that enzyme treatment was effective in increasing oil recovery.

Oil recovery increased 128 and 45% for the blended CCDS subjected to hydrolysis with the enzyme combination and alkaline protease alone, respectively, compared to no-enzyme controls. For the CCDS which was not subjected to blending, the increase in oil recovery was only 26 and 15% after using enzyme combination and alkaline protease, respectively, suggesting that particle size reduction by blending also greatly increased enzyme efficiency.

When CCDS was ground using a mortar and pestle to decrease particle size of the large CCDS particles, oil recovery significantly increased for the non-enzyme treated ground samples, 82 versus 78% (Table 4). When enzymes

Table 3 Effects of blending and enzyme hydrolysis on oil recovery from CCDS

Treatment	% Oil recovery ± SD	
	Without blending	With blending
Multifect® CX GC cellulase + Multifect® AA 21 L α-amylase + Alcalase 2.4L alkaline protease (pH 4 and then 9)	64.5 ± 1.5ab	48.7 ± 8.7bc
Alcalase 2.4L (pH 9)	67.9 ± 0.2a	59.2 ± 0.2ab
No enzyme (pH 9)	59.1 ± 4.3ab	40.8 ± 7.6bc
No enzyme (pH 4 and then 9)	51.2 ± 21.5bc	21.4 ± 7.7d

Enzyme dosage (5% v/w) was based on solids content of CCDS. Means followed by different letters are significantly different ($P < 0.05$)

Table 4 Effects of grinding CCDS on oil recovery

	% Oil recovery ± SD	
	Without grinding	With grinding
Multifect® Pectinase FE pectinase + Protex™ 13 FL acid protease	80.7 ± 1.0ab	82.6 ± 0.9a
No enzyme	78.4 ± 2.4b	82.2 ± 1.4a

Enzyme dosage (5% v/w) was based on solids content of CCDS. Means followed by different letters are significantly different ($P < 0.05$)

hydrolysis was performed using the combination of Multifect® Pectinase FE and Protex™ 13FL acid protease, oil recovery did not significantly increase, 83 versus 82% for enzyme treatment with grinding and no enzyme with grinding. Since grinding already significantly increased oil recovery, enzyme hydrolysis did not further increase oil recovery. The increased oil recovery after grinding suggests that some of the oil was present in the large endosperm or germ particles and could not be recovered by centrifugation alone. Since about 20% oil could not be recovered, such oil may be in the finer unbroken particles, and/or attached to hydrophobic protein and cell wall components as small oil droplets that are difficult to flocculate and separate by centrifugation.

Verification of Interaction of Oil with Hydrophobic Protein

The interaction between the hydrophobic protein zein and oil was strong in the model system as evidenced by only 10% oil recovery with centrifugation alone as shown in Table 5. When Alcalase 2.4L alkaline protease was used to hydrolyze the protein, oil recovery increased to 97% and only 3% of the oil remained complexed to zein. Heating resulted in an even lower oil recovery because hydrophobic interactions between the oil and zein were made stronger by increasing temperature. When the CCDS protein was targeted for hydrolysis using the same protease, the oil recovery was not as high as in this model, at most 70%. Therefore, the freed oil may have been attached to other surfaces or may be present as minute droplets. This model experiment was only intended to provide evidence of the strong hydrophobic interaction between oil and protein. In reality and with complex system the zein protein may not be easily hydrolyzable or the freed oil may be finely dispersed and could not be easily separated.

Table 5 Oil recovery from zein and oil dispersion by various treatments

Treatment	% Oil recovery ± SD
Alcalase 2.4L alkaline protease	97.3 ± 0.2a
Heating at 100 °C	1.8 ± 0.8c
Control	10.1 ± 0.3b

Enzyme dosage (5% v/w) based on the solids content of zein + oil dispersion. Means followed by different letters are significantly different ($P < 0.05$)

The protein content of the corn kernel ranges from 6 to 18%, and since the endosperm occupies a large fraction of the corn kernel, it contains 75% of the total kernel protein [21]. The major storage protein of the corn endosperm is zein, constituting up to 79% [21]. Therefore, zein may have a major contribution to oil and protein interaction in the CCDS matrix.

Transmission Electron Microscopy (TEM) Imaging of CCDS

TEM was done on original CCDS, CCDS that had been subjected to the blending treatment, and the CCDS residue after blending, enzyme hydrolysis and centrifugation to remove free oil. The following interpretation was provided by experts at the TEM facility at Iowa State University. Figure 2a shows the distribution of lipid droplets as dark spheres and the lipid droplets are surrounded by dispersed protein. The proteins are the dense network giving a granular appearance to the cytoplasm. The protein was denatured because of the heat treatment during ethanol distillation. Intact cell walls were present with two cells attached to each other. Therefore, CCDS has intact cells possibly from the large pieces of endosperm but mainly the unbroken germ and this observation may partially explain why some oil cannot be recovered.

Figure 2b shows the CCDS that was reduced in particle size in an attempt to improve the effectiveness of enzyme hydrolysis. This CCDS sample shows broken cell walls and no intact cells. The lipid droplets became trapped in the protein network and also attached to the cell wall. Even though reducing CCDS particle size by blending may be effective in breaking the intact cell, the high agitation force results in the lipid droplets becoming trapped in the protein matrix and broken cell debris.

Figure 2c shows that after subjecting the blended CCDS to enzyme hydrolysis and centrifugation to remove the free oil, the dense cytoplasm of the original CCDS disappeared indicating protein hydrolysis by protease. There were no intact cell walls indicating that cellulase had degraded the cell walls. The lipid droplets appear not to be complexed to protein or cell wall debris. Interestingly the lipid droplets

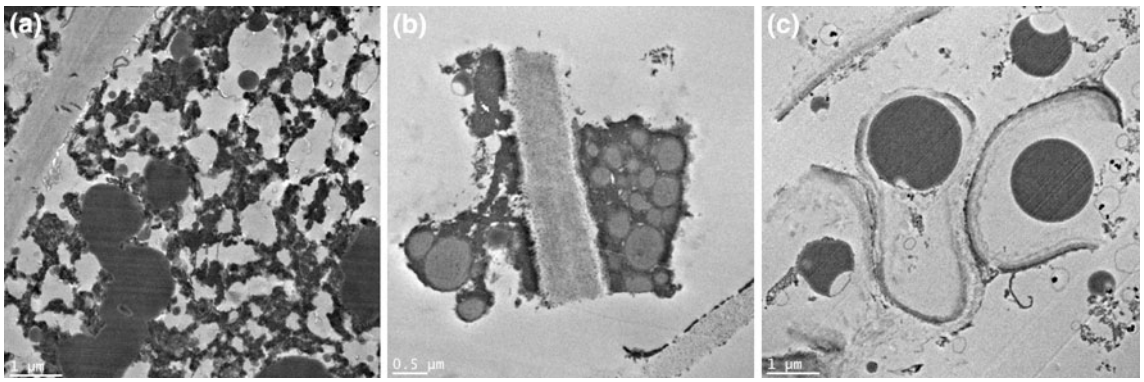

Fig. 2 Transmission electron microscopy (TEM) images of CCDS. **a** Original CCDS showing lipid droplets (*dark spheres*) dispersed throughout the cell, some lipid droplets interacting with the protein. **b** CCDS after blending showing lipid droplets trapped in the protein matrix and degraded cell walls. **c** CCDS residue after enzyme hydrolysis using cellulase, α-amylase and alkaline protease showing free lipid droplets

could not be recovered by centrifugation. Blending reduced the size of oil droplets which were difficult to float in a viscous CCDS matrix. Therefore, churning CCDS may bring about coalescence of these small oil droplets so that the oil can float and be easily separated by centrifugation.

Effects of Enzyme Hydrolysis and Centrifugal Force on Oil Recovery

Centrifugal force was expected to have a significant effect on oil separation from the CCDS. A pectinase mixture and protease were used together for the enzyme hydrolysis before centrifuging at 8,500–12,240×g, and the oil recovery ranged from 78 to 81%. Unexpectedly, oil recovery was not significantly affected by increased centrifugal force. This result indicates that after enzyme treatments, centrifuging at relatively low speed is sufficient to separate the liberated oil. Since the enzyme treatments may have liberated the oil from the oil-in-water emulsion, oil bodies, and oil bound to cell wall material, increasing centrifugal force beyond a critical speed would not further increase oil recovery. Our results for the control samples showed that centrifugation slightly increased oil recovery when no enzymes were used.

It should be noted that oil recoveries in this study were based on the total oil content in CCDS determined by the standard acid hydrolysis procedure. It is well known that this procedure gives considerable higher oil content than the polar solvent extraction oil quantification method [22] and with the Folch wash [23]. Therefore our oil recovery may be underestimated if compared with other solvent extraction results.

Conclusions

Increasing protease concentration slightly increased oil recovery, indicating hydrolysis of protein destabilized the oil-in-water emulsion in the CCDS matrix. When the protease was used in combination with a cellulase, oil recovery was greater compared to protease alone. CCDS contains unbroken germ particles, and grinding CCDS significantly increased oil recovery for the non-enzyme treated samples. Particle size reduction by blending increased enzyme efficiency but oil recoveries were lower than for unblended CCDS. Hydrophobic protein zein may contribute significantly to oil and protein interactions thereby stabilizing the oil in the CCDS matrix as evidenced in the zein and oil model system.

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