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Oil recovery from condensed corn distillers solubles

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ABSTRACT

Condensed corn distillers solubles (CCDS) contains more oil than dried distillers grains with solubles (DDGS), 20 vs. 12% (dry weight basis). Therefore, significant amount of oil is present in the liquid fraction after fermentation and ethanol distillation. The oil removed represents a significant alternative feedstock for biodiesel production. The objectives of the present research were to study the effect of enzyme hydrolysis on oil recovery from CCDS, to determine the effect of physical and chemical processes on oil recovery from CCDS, and to characterize quality of oil recovered from CCDS and the nature of deposits in CCDS oil. Employing enzyme processes with Protex ™ 13FL (acid protease) increased oil recovery as enzyme concentration increased, with greatest oil recovery, 70% being achieved at 10% v/w (dry weight basis) enzyme concentration. Reducing the particle size of CCDS (by grinding) increased oil recovery, achieving 83% when Multifect® Pectinase and Protex™ 13 FL were used. Zein-lipid interaction in a model system was strong such that only 10% of the oil could be freed by centrifugation alone. Following enzyme hydrolysis of the zein-oil complex with Alcalase 2.4L, oil recovery increased to 97%. For the physical and chemical processes, heating increased oil recoveries, 2.5-fold when temperature was increased from 25 to 59 °C. Oil recovery at acidic pH was significantly greater than at alkaline pHs. Oil extraction using isopropanol and butanol achieved > 80% total oil recovery. When oil was co-extracted with zein using hexane as a co-solvent, greatest total oil recovery achieved was 89%. Churning CCDS for 3 h at 50 °C and pH 3.5, up to 80% of the oil could be recovered. CCDS oil contains lipids (CCDS oil deposit) that solidify and settle to bottom of tank at ambient temperatures. This deposit had high free fatty acid (36%), high palmitic acid and wax contents causing a semi-solid appearance at ambient temperatures.

CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH JUSTIFICATION

The primary feedstock used for the production of fuel ethanol is corn (maize). The dry-grind process is the most widely used method employed because of low capital investment, process simplicity and high ethanol yield (Singh and Cheryan, 1998). The dry-grind process represented 82% of the industry in 2006 (Renewable Fuels Association, 2007) whereas the wet-milling process represented 18%. The dry-grind process results in a single co-product, dried distillers grains with solubles (DDGS). The dry-grind process involves cleaning, grinding, cooking, enzymatic hydrolysis, fermentation, ethanol stripping, centrifugation to separate the thin stillage and the solids (wet distillers grains, WDG), water evaporation from the thin stillage, combining the concentrated solubles (syrup) with the WDG and drying the mixture with a drum dryer to give DDGS. At least 26% and as much as 50% of the thin stillage, referred to as "backset", is recycled back to the liquefaction stage for conserving water, buffering pH and providing yeast nutrients (Kwiatkowski et al., 2006; Maisch, 2003) and the remainder is evaporated to form the concentrated syrup (condensed corn distillers solubles).

Currently, condensed corn distillers solubles (CCDS) or syrup is mixed with DDG to give DDGS as a valuable feed co-product. Normally CCDS contains about 65% moisture, 14% protein, and 20% total oil on a dry weight basis. DDGS contains about 11% moisture, 30-31% protein, and 11-12% total oil (Spiehs et al., 2002; Belyea et al., 2004). CCDS contains more oil compared to DDGS (20 vs. 12%) on a dry weight basis. Since DDGS has high protein and fiber contents, it is utilized as a feed ingredient by the beef and dairy cattle industries. Small amounts are used for swine and poultry feeding. Problems with the high oil content in DDGS have been recognized such as causing softer belly fat in pigs (Whitney et

al., 2006) and the presence of high levels of unsaturated fatty acids interferes with normal milk fat production in dairy cattle (Da Cruz et al., 2005), although higher oil content leads to increased milk production. Therefore removal of the oil would improve feed quality and increase demand by livestock feeders. The challenge is to find ways to efficiently and cost-effectively remove the oil. The oil represents a significant alternative feedstock for biodiesel production. The oil is dark-colored, viscous and high in free fatty acid content, which are indicators of oil degradation and therefore oil is unsuitable for human consumption.

About 23 million metric tons of DDGS were produced in 2008 as reported by the Renewable Fuels Association (2009) and industry experts predict that this will increase due to rapid industry expansion. This amount translates to about 2.76 million metric tons of oil in the corn ethanol co-product. Assuming 70% of this oil could be recovered, about 2.2 billion liters (547.6 million gallons) of biodiesel can be made from this additional corn oil. In addition, oil has a higher price than DDGS ($500/ton vs. $100 - $160/ton), suggesting that the removal of oil from corn fermentation co-products will be profitable to the ethanol industry (Singh and Cheryan, 1998).

Several strategies have been explored to recover oil in the dry-grind ethanol plants. Oil from corn ethanol production can be recovered by front-end degerming used in wet-milling process (Kwiatkowski et al., 2006). Most of the ethanol production uses the dry-grind process in which the corn kernel is ground in a hammer mill, cooked, subjected to enzyme hydrolysis, and then fermentation to make ethanol (Renewable Fuels Association, 2007). In this process, oil can be recovered either at the front-end by dry mill degerming or from the co-products after ethanol distillation. Oil recovery from corn fermentation co-products has been ineffective (Cantrell and Winsness, 2006). The recovery of oil from thin stillage before

evaporation has been attempted by centrifugation. Centrifugation of the thin stillage which contains 5-10% solids does not produce significant amounts of utilizable oil (Winsness et al., 2007). The oil becomes trapped as an emulsion which needs to be broken to release the oil requiring additional processing (Winsness et al., 2007). In addition, since thin stillage has a low solid content, its volume is 2 to 10 times greater than CCDS and large capacity centrifuges would be required for oil separation making this process expensive and inefficient (Winsness et al., 2007).

Recovering oil from CCDS may be more efficient and economical than from thin stillage. Oil recovery from CCDS can be achieved by several means including: enzyme hydrolysis of interfering substances, solvent extraction, and pH changes. The recovered oil from CCDS can be separated by gravity separation, by means of a settling tank (the oil is allowed to naturally to rise to the top for recovery) or centrifugation, or by droplet separation with micro/ultrafiltration (Winsness et al., 2007). Centrifugation is regarded as the best option for separating the released oil because it is a continuous process and the CCDS is split into three phases; a solid heavy phase, a water intermediate phase, and an oil-rich light phase (Winsness et al., 2007). The oil phase is then removed and the water can be used as wash water and the solid phase combined with WDG. In a settling tank, the oil is allowed to rise to the top of the CCDS. One major drawback is that when oil droplets are small, they will not float. Continual mixing favors formation of large oil droplets (coalescence) increasing release of oil. Microfiltration or ultrafiltration has problems with frequent clogging of the filters and increased operation costs (Winsness et al., 2007).

CCDS is a stable matrix and the oil is difficult to be extracted by centrifugation alone. We believe that the oil is present in four forms based on our observations:

1. Oil-in-water emulsion stabilized by emulsifiers such as protein, free fatty acids, mono and di-glycerides and phospholipids;
2. Oil complexed to the surface of fibrous materials and of hydrophobic protein zein;
3. Oil bodies in the unbroken germ or endosperm particles with intact cell structure; and
4. Oil bodies released from the broken germ and endosperm.

The overall goal of the present research was to enhance or maximize oil recovery from CCDS. In the first and second studies, the effects of enzyme and physical and chemical processes such as heating, pH changes, particle size reduction and solvent extraction on oil extraction yield from CCDS were evaluated. The third study characterizes the deposit in oil derived from CCDS recovered oil.

Dissertation organization

The present dissertation is comprised of a general introduction, literature review, three papers and a general conclusion. The literature review focuses on dry-grind processing for ethanol production, composition and applications of the co-products, oil composition of corn and oil recovered from corn fermentation co-products.

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CHAPTER 2. LITERATURE REVIEW

Dry-grind ethanol process

The dry-grind ethanol production process is the most widely employed method used by fuel ethanol production industries because of its simplicity and low capital investments. In 2006, 82% of the production employed the dry-grind process for ethanol production whereas the wet-milling process represented 18% (Renewable Fuels Association, 2007). In the wet-milling process, the germ and fiber are separated before the corn kernel is subjected to starch saccharification and fermentation (Kwiatkowski et al., 2006). Dry-milling plants are smaller in size and primarily produce ethanol and feed only while wet-milling facilities are called corn refineries because they also produce high valued co-products, high fructose corn syrup and glucose syrup (Lee, 2007). The overview diagram of the ethanol production process is shown in Figure 1 and 2.

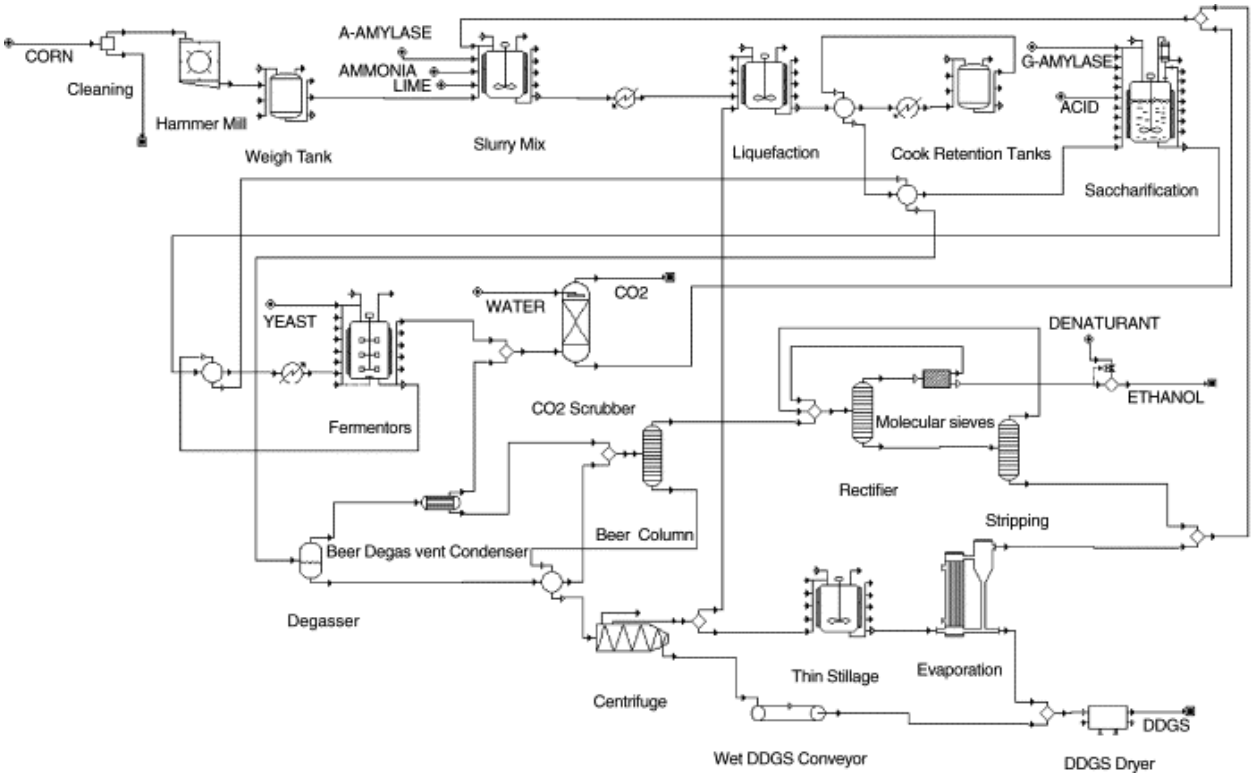

Figure 1. Dry-milling process for ethanol production from corn **(**Kwiatkowski et al., 2006).

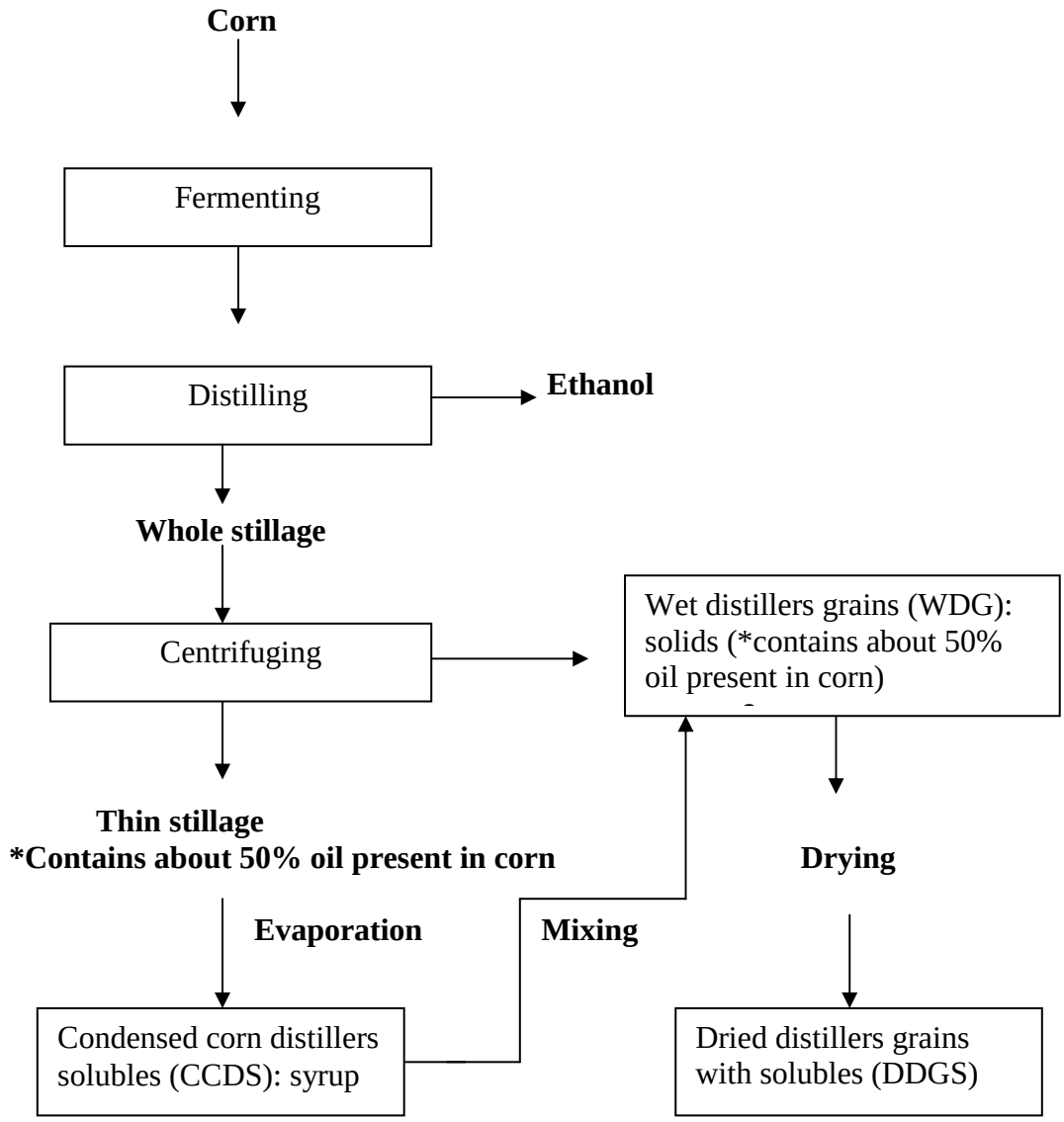

Figure 2. Dry-milling process for ethanol production from corn.* Based on our calculation using composition of commercial products.

Corn receiving and grinding: The corn is received and stored in silos for no more than 12 days prior to cleaning. The grain composition is evaluated for moisture, protein, starch, and fat contents and is also screened for damaged kernels, foreign matter, mold toxins and contaminants (Maisch, 2003). These values are used to determine grade and value of incoming feedstocks. The corn is then ground by using hammer mills into a meal to an

average particle size diameter of 0.94 mm (Rausch et al., 2005). Generally, the choice of particle size of the meal is influenced by the type of cooking that will be used, atmospheric (100 °C) vs. pressure cooking (145 °C). Pressure cooking requires that the particles of the meal be medium coarse. The particle size of the ground corn has an effect on the rate of fermentation, the solids content in whole stillage, and the separation of the solids from the liquid material during centrifugation (Maisch, 2003). If the corn is present in small particles due to fine grinding, this can lead to poor fermentation efficiency and leave residual sugars. In addition, the fines will become part of the thin stillage fraction and excessive fines can negatively affect evaporation of the thin stillage to syrup as they take the consistency of peanut butter (Maisch, 2003).

Liquefaction, saccharification and fermentation: The process that converts the starch to fermentable sugars is the mashing step which includes liquefaction and saccharification. The ground corn is mixed with water to give about 30% solids, heat stable α-amylase (0.082% d/b) is added, ammonia and lime are added at 90 kg/hr and 54 kg/hr respectively to form a slurry (Kwiatkowski et al., 2006) for starch hydrolysis in a continuous system. The amount of water added depends on requirements for downstream operations, stirring and pumping (Maisch, 2003). In the initial step, the starch granules imbibe water, and when heated the starch granules swell, hydrogen bonds are broken and the starch becomes gelatinized. At this stage the slurry becomes thick and heat stable α-amylase is important for hydrolyzing the starch molecules to oligosaccharides by breaking the α-1,4-glycosidic linkages of starch, thereby thinning the slurry at 105-107 °C (Maisch, 2003). This is accompanied by using a jet cooker and then holding the slurry at 95 °C for 2 h in a process called liquefaction (Maisch,

2003). The output from the liquefaction step is mixed with a portion of thin stillage/ "backset" (Maisch, 2003). The thin stillage is obtained after the whole stillage has been stripped of carbon dioxide and ethanol and has undergone centrifugation to produce wet distillers grains and thin stillage. The thin stillage provides nutrients to the yeast and is also important for water conservation and pH adjustment.

Following liquefaction, glucoamylase (0.11% db) is added for the further conversion of oligosaccharides to glucose in a process called saccharification (Kwiatkowski et al., 2006). The enzyme cleaves glucose molecules from the non-reducing ends of the oligosaccharides or dextrins. The enzyme also hydrolyzes α-1,6-glycosidic linkages at a slower rate. Saccharification is conducted at pH 4.5, and 61 °C for 5 h. Sulfuric acid is added to lower the pH to maximize glucoamylase activity. The slurry is transferred to a fermentation tank after saccharification and cooled to 32-35 °C prior to fermentation. In the fermentation process, yeast converts the glucose to ethanol and carbon dioxide. The fermentation is typically carried out for 68-72 h, and continuous cooling is done throughout fermentation because heat is produced during the process, about 516 BTU (British Thermal Unit) (544 kJ) of heat per pound of ethanol (Grethlein and Nelson, 1992). The stoichiometric yield of the fermentation process can be shown by the following formula:

i. Starch ($C_6H_{10}O_5$, 162 g/mol) + water (H_2O, 18 g/mol) = glucose ($C_6H_{12}O_6$, 180 g/mol) (Maisch, 2003)
ii. Glucose ($C_6H_{12}O_6$, 180 g/mol) = 2 ethanol (C_2H_5OH 92 g/mol) + 2 carbon dioxide (CO_2, 88 g/mol) (Maisch, 2003)

According to the above stoichiometry, if we have 100 kg of corn which contains 70 kg of starch on a dry weight basis, the starch is converted to glucose yielding 78 kg of

glucose. Theoretically, the 78 kg of glucose will yield 40 kg (51% w/w) of ethanol and 38 kg (49% w/w) of carbon dioxide and the non fermentable material, distillers grains are left (Maisch, 2003). The yeast uses about 5% w/w of the glucose for the production of yeast cells during fermentation (Kwiatkowski et al., 2006).

Distillation and dehydrating: Distillation using a continuous downward passing from plate to plate and discharges at the bottom follows fermentation (Maisch, 2003). The alcohol is stripped from the fermented slurry by distillation using heat from steam. Steam provides heat to boil off the ethanol in the distillation column. The steam is directly mixed with the fermented slurry. The alcohol vapors are flashed from the top of the column and condensed in a cooled condenser. Uncondensed vapors are combined with carbon dioxide produced during fermentation and sent through the carbon dioxide scrubber. Fuel grade ethanol should not contain > 0.5% water. Alcohol-water mixtures form a boiling mixture which is azeotrope mixture having 95.4% wt ethanol and 4.6% wt water. The remaining water cannot be removed by ordinary distillation (Lee, 2007). Therefore, in order to produce water free ethanol, additional steps are required following distillation. Benzene can be added to the ethanol-water mixture and changes the boiling characteristics of the solution allowing separation of the anhydrous ethanol in a process called azeotropic distillation (Lee, 2007), but this older method has been replaced by more efficient molecular sieves. Molecular sieves are currently used and they selectively adsorb water inside micro-porous beads and the large ethanol molecules flow around them (Kwiatkowski et al., 2006) resulting in 99.6% pure ethanol. The water is recovered when the molecular sieves are regenerated and this water is added to the slurry of incoming ground corn (Kwiatkowski et al., 2006).

The anhydrous ethanol is then blended with about 5% denaturant (such as unleaded gasoline) to render it undrinkable and thus not subject to beverage alcohol tax (Renewable Fuels Association, 2009a). It is then ready for shipment to gasoline terminals or retailers. Currently, one bushel of corn (56 pounds) produces approximately 2.8 gallons of ethanol and more than 17 pounds of distillers grains (Renewable Fuels Association, 2009a).

Centrifugation of whole stillage: The unfermented material containing about 15% solids from the beer column at high temperature is fed to the whole stillage tank prior to centrifugation (Kwiatkowski et al., 2006). Whole stillage contains the protein, fiber, fat and ash of the original corn. About 83% of the water present in the whole stillage is removed by centrifugation as part of thin stillage to produce wet distillers grains (WDG) containing about 37% solids (Kwiatkowski et al., 2006). Part of the thin stillage, at least 26% and as much as 50% is used as a backset and is combined with the slurry in the liquefaction step and the rest goes to the thin stillage tank (Maisch, 2003). Small particles from the fine grinding increase the solids content of the thin stillage (Maisch, 2003). The thin stillage is fed to multiple-effect evaporators where water is removed and recovered. The 4-effect evaporator uses overhead vapors from the rectifier instead of steam to provide heating for the first-effect evaporator (Kwiatkowski et al., 2006). The concentrate (CCDS) contains 30-35% solids (Kwiatkowski et al., 2006).

The compositions of the CCDS and distillers grains are not the same. The CCDS contains some yeast cells and soluble nutrients in addition to the fat, fiber and protein. The CCDS is mixed with the WDG coming from the centrifuge and then the mixtures are passed to a rotary drum dryer (Kwiatkowski et al., 2006). Consequently, the heated streams provide

heat for the following effects of evaporation stages. The moisture content is reduced from 64 to 10% to produce the dried distillers grains with solubles (DDGS) (Kwiatkowski et al., 2006).

Corn ethanol fermentation co-products

The corn fermentation process to produce fuel ethanol uses the carbohydrate portion of the grain and the other components, protein, fiber and oil become non-fermentable portion and are included in the co-product stream (Ganesan et al., 2006). The non-fermentable materials are obtained as whole stillage after ethanol distillation and the carbon dioxide has been vented off. As described above, the whole stillage undergoes centrifugation to produce the WDG and thin stillage. The thin stillage is concentrated by removing water when passing through evaporators to produce the CCDS. The CCDS is then mixed with the WDG to make the wet distillers grains with solubles (WDGS) which can be dried to produce the DDGS.

Dried distillers grains with solubles: Currently DDGS is used as a feed ingredient mainly in dairy and cattle, 42%, whereas swine and poultry represent 11 and 5%, respectively, of the total DDGS utilized as feed ingredient (Renewable Fuels Association, 2007). The high fiber content limits the use of DDGS in monogastric livestock (Kim et al., 2008). The production of DDGS continues to increase and in 2008, 23 million metric tons were produced (Renewable Fuels Association, 2009b). This was an increase of approximately 36% from 2007.

The sale of DDGS to the feed industry contributes to the economic viability of the ethanol producing industries (Ganesan et al., 2006). The composition of DDGS has been

extensively reported (Kim et al., 2008; Spiehs et al., 2002; Belyea et al., 2004). DDGS contains approximately, 88% dry matter, 25-31% protein, 11-12% crude lipids, 9-10% crude fiber, 5% starch, 16-17% acid detergent fiber and 5-6% ash on a dry weight basis. The value of DDGS is mainly based on the protein content (Kwiatkowski et al., 2006). When DDGS was fed to pigs, at 30% diet inclusion, the belly fat became more unsaturated and soft, and this may affect further processing traits (Whitney et al., 2006). This was attributed to the unsaturated fatty acid content in the DDGS.

Condensed corn distillers solubles: CCDS is a viscous liquid which resembles syrup. It is an excellent source of fat, minerals and protein (Maisch, 2003). The CCDS contains 28 to 46% solids, 6 to 21% crude lipid, 18 to 22% protein, and 9 to 12% fiber on a dry weight basis (Belyea et al., 1998). CCDS has been used as a feed ingredient for beef cattle and lactating dairy cows. Since the energy to dry WDGS to DDGS is a major cost factor, efforts have been made in using wet CCDS as a feed ingredient for both beef and dairy cattle.

Milk yield increased when lactating Holstein dairy cows had 5 and 10% (dry basis (db)) CCDS as a feed ingredient in their diets over a five week period with no statistical significant difference at the higher CCDS level and this was attributed to the increased fat content in the CCDS diets, 4.3% fat for the 5% CCDS diet, 5.2% fat for the 10% CCDS diet vs. 3.4% fat for the control diet (Da Cruz et al., 2005). Milk yield was increased by 4.1 and 5.0% for the 5 and 10% CCDS, respectively, when compared to control diet (Da Cruz et al., 2005). For the fatty acid compositions, long-chain fatty acids in the milk fat increased, whereas medium-chain fatty acids decreased, no change was observed for the short-chain and saturated fatty acids content in the CCDS diets (Da Cruz et al., 2005). In addition, the protein

and lactose contents of the milk increased, however, the milk fat percentage decreased slightly in the CCDS diets (3.1 - 5.9%) compared to control diet (Da Cruz et al., 2005). Therefore, CCDS as a feed ingredient for dairy cows may increase milk yield but may also alter milk fat percentage.

Mixed-ration diets for lactating Holstein cows containing 10 and 20% CCDS resulted in an average 7% increase in milk yield (Sasikala-Appukuttan et al., 2008). The fat content contributed by CCDS in the CCDS diets was 2 to 4% (Sasikala-Appukuttan et al., 2008). A similar trend was observed in which the long-chain fatty acids concentrations increased, whereas the medium-chain fatty acids concentrations decreased (Sasikala-Appukuttan et al., 2008). Changes in the conjugated linoleic acid (CLA) were also observed in the milk fat fatty acid composition (Sasikala-Appukuttan et al., 2008). The *cis* 9, *trans* 11 CLA increased by 55 and 158 % for the 10 and 20% CCDS diets, respectively, while *trans* 10, *cis* 12 CLA doubled for the 20% CCDS diet (Sasikala-Appukuttan et al., 2008). This is highly desirable as CLA have been purported to have health benefits.

Supplementing low-quality forages based diets with CCDS for steers increased dry matter intake, fiber digestion, total tract crude protein digestibility and microbial crude protein synthesis at levels up to 15% (Gilbery et al., 2006). Incorporating CCDS in animal feed can be done up to 20% to provide the needed crude protein, lipids and other essential nutrients. The lipid content when CCDS is used as a feed ingredient usually increases by 1 to 4%. Oil content of the CCDS is the limiting factor resulting in up to 20% CCDS being used as a feed ingredient in dairy cattle. Since CCDS contains 20% total lipid content, removing some of the oil increases the protein content, and as a result higher concentrations of the CCDS may be used in animal feed diets.

Corn oil composition

The oil extracted from co-products of dry-grind corn ethanol production is from the whole corn kernel and would have the lipid constituents of germ, endosperm, bran, fiber and yeasts. Today`s commercial corn hybrids contain about 4.2% oil (White and Weber, 2003) with 80% of this in the germ (Gunstone and Harwood, 2007). The crude corn oil composition from germ has been reported (Orthoefer et al., 2003) and is shown in Table 1. Corn oil contains 1.3 to 2.3% unsaponifiables and these include free and esterified sterols, tocopherols and hydrocarbons such as squalene (Gunstone and Harwood, 2007). Total sterols include β-sitosterol, campesterol, Δ^5 avenosterol and stigmasterols (Gunstone and Harwood, 2007).

Corn fiber oil is unique in that it is a rich source of ferulate sterol esters such as those found in rice bran oil (Moreau et al., 1996). The corn fiber oil has shown to lower blood cholesterol in an animal model. Therefore corn fiber oil is acclaimed as an important nutraceutical (White and Weber, 2003). Corn fiber extracted using hexane gave 3.3% extractable oil of which 4.95% was ferulate esters, 9.1% was phytosterol esters, 1.0% was free phytosterols, and 79% triacylglycerols (Moreau et al., 1996).

The fatty acid composition of corn oil consists of about 11% palmitic, 2% stearic, 28% oleic, 58% linoleic, and 1% linolenic. Hydrocarbons, polyisoprenoid alcohols are some of the compounds in corn oil (White and Weber, 2003). Crude corn oil also contains waxes. The corn kernel waxes are composed of mainly wax esters (Bianchi and Avato, 1984). The composition of the esters from maize kernel wax were comprised mainly of 46, 48, 52 and 54 carbon chain length and the predominant esterified fatty acids were C22, and C24 whereas the esterified alcohols were C22, C24, C26 and C32 (Bianchi and Avato, 1984). The maize

kernel wax fraction was comprised of mainly of 6% alkanes, 2% alcohols, 11% acids, 76% esters, and 5% sterols (Bianchi and Avato, 1984).

Oil bodies in mature corn: Triacylglycerols (TAG) are the energy reserve for germinating or post-germination seedlings (Tzen and Huang, 1992). TAG is present in spherical oil bodies of approximately 1 µm in diameter in the cells of plant seeds (Huang, 1996). The average diameter of maize oil body is 1.45 µm (Tzen and Huang, 1992). Oil seed oil bodies are surrounded by a layer of phospholipids and then a layer of unique proteins called oleosins (Huang, 1996). These play a structural role and maintain the integrity of the oil bodies. Isolated maize oil bodies contained mostly TAG (97.7%), phospholipids (PL) (0.9%), and protein (1.4%) (Tzen and Huang, 1992).

Enzyme hydrolysis of maize oil bodies with trypsin showed that the oleosins were hydrolyzed into smaller polypeptides which were revealed by SDS-PAGE (Tzen and Huang, 1992). However, when phospholipase A2 and C were used to hydrolyze the PL in the maize oil body, little to no hydrolysis took place because of the inaccessibility of the enzymes to the PL due to the shielding effect of oleosins (Tzen and Huang, 1992). These all have implications on how to recover the corn oil from the corn fermentation co-products.

Yeast lipid composition

The yeast species, *Saccharomyces cerevisae* is used in the corn fermentation process and contains about 9% (db) total lipids (Rattray, 1988). The lipid composition for a batch culture of the yeasts is 40% triacylglycerols, 6% free fatty acids, 20% sterol esters, and 30% phospholipids (Rattray, 1988).

The yeasts *Saccharomyces cerevisae* fatty acid composition of the triacylglycerol fraction includes 3% myristic (14:0), 16% palmitic (16:0), 42% palmitoleic (16:1), and 27% oleic (18:1) (Rattray, 1988). The growth conditions of the yeasts and nutrients have an impact on the fatty acid composition of the yeasts. For example, yeast growing in a medium containing palmitoleic acid (16:1) would result in 16:1 becoming 91% of the total fatty acid composition and supplementation with oleic acid (18:1) resulted in 18:1 becoming 90% of the total fatty acid composition (Keith et al., 1973). Therefore, yeasts are able to incorporate lipids from the natural media in which they are grown (Beltran et al., 2008). It is only in the absence of natural media (medium without lipids) that the final lipid composition of yeasts reflects the changes that occur during yeast metabolism (Beltran et al., 2008).

The phospholipids composition of whole yeast cells (2.7% total phospholipids) includes 48% phosphatidylcholine (PC), 23% phosphatidylethanolamine (PE), 16% phosphatidylinositol (PI), 7% phosphatidylserine (PS), and 3% cardiolipin (Suomalainen and Nurminen, 1970). However, for the corn dry-grind ethanol production process, the amount of yeast obtained at the end of fermentation has not been estimated. Therefore, contribution of yeast lipids to the total oil in fermentation co-products is unknown.

Oil recovery from corn fermentation co-products

There has been little published research on oil recovery from corn fermentation co-products. Current industry practices on oil recovery from corn fermentation co-products gives 0.6 pounds of oil per 56 pounds (1 bushel) of corn (*personal communication*). This is equivalent to approximately 25% oil recovery. Some work has been done on the extraction of oil from DDGS and only approximately 50% crude oil could be extracted from DDGS using

6:1 ethanol-to-DDGS ratio in a single-stage extraction (Singh and Cheryan, 1998). Randhava et al. (2008), in their patent described oil recovery from corn fermentation co-products by using ethyl acetate and isopropranol acetate. They also described a process in which the milled corn is extracted of oil prior to corn ethanol fermentation process such that oil-free co-products are produced, and oil recovery from CCDS was at least 99%, irrespective of solvent used (Randhava et al., 2008). In another patent, Janes et al. (2007) described a method of using hexane to extract oil from DDGS to achieve at least 77% oil recovery. Although these oil recovery processes results in substantial amount of oil being recovered from corn fermentation co-products, new infrastructure and substantial capital investment would be required and adding new processing equipment would be costly for the dry-grind ethanol industry at a time when the ethanol industry is economically challenged.

Winsness et al. (2007) suggested using high temperature, 100-121°C and pressure up to 552 kPa (80 psi) on CCDS in order to free most of the bound oil, however, the authors did not mention the length of time the CCDS was subjected to under these conditions and how much oil was obtained except that substantially all the oil was recovered. The CCDS suspended solids were hydrolyzed (conversion of suspended solids to dissolved solids) releasing bound oil. These experimental conditions are extreme and may not be attainable in the dry-grind corn ethanol process.

One feasible way to remove oil from CCDS is by centrifugation (Cantrell and Winsness, 2006). This can be done at the decanting step of the process for separating solids from the liquid after ethanol distillation, or as an additional centrifugation step after necessary treatments to free the oil. In the current industry process, 50% of the total oil becomes part of the thin stillage (liquid fraction) and the remainder goes to the solids fraction

(wet distillers grains) (Wang et al., 2008). Therefore, in order to maximize oil recovery by centrifugation it may be desirable for most of the oil to partition to the liquid fraction so that it may then be subjected to different treatments to make oil separable by centrifugation (Wang et al., 2008). Since CCDS appears to be a stabilized matrix because of the mixing, shearing, cooking and heating during the ethanol production process, oil recovery by centrifugation alone is low. Therefore, in the present research we have examined the effects of enzyme hydrolysis of interfering substances, and physical and chemical processes on oil recovery from CCDS.

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Table 1. Lipid composition of corn

Component	[a]Crude oil from corn germ (%)	[b]Crude oil from corn kernel (%)	[c]Corn fiber oil (%)	[d]Yeasts lipids
Triacylglycerol	95.6	76	79	40
Diacylglycerol	-	2.1	-	-
Free fatty acids	1.7	1.1	-	6
Waxes	0.05	-	-	-
Phospholipids	1.5	13	-	30
Total phytosterols	1.2	4.6	15	20 (sterol esters)
Hydrocarbons	-	3.4	-	-
Tocopherols	0.06	-	-	-

[a]Orthoefer et al., 2003
[b]Weber, 1969
[c]Moreau et al., 1996
[d] Rattray, 1988
-Not reported

CHAPTER 3. ENZYME TREATMENTS TO ENHANCE OIL RECOVERY FROM CONDENSED CORN DISTILLERS SOLUBLES

A manuscript submitted to *Bioresource Technology*

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Abstract

The objective of this present study was to determine the effect of enzyme hydrolysis of various corn components on oil recovery from condensed corn distillers solubles (CCDS). Hydrolysis with Protex™ 13FL (a commercial acid 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 x *g* was only slightly effective for the non-enzyme treated samples. Reducing CCDS particle size by grinding increased oil recovery to 83% when an enzyme combination of Multifect® Pectinase and Protex™ 13FL was used. Particle size reduction of CCDS by blending resulted in low oil recovery, but the percentage of oil recovery improvement was significant 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 Alcalase 2.4L alkaline protease, oil recovery was increased to 97%. Overall, enzyme hydrolysis and further particle size reduction showed promise in increasing oil recovery from CCDS.

Keywords: CCDS, protein-lipid interactions, carbohydrate-lipid interactions, oil recovery, corn oil.

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Introduction

Condensed corn distillers solubles (CCDS) typically contains 35% solids, 14% protein, and 20% oil on a dry weight basis. When comparing to dried distillers grains with solubles (DDGS), CCDS contains more oil, 20 vs. 12% w/w. We are currently also developing other processing means to have more oil partitioned in the liquid fraction so more oil can be recovered from the CCDS. Such oil presents an alternative source for biodiesel production. However, there are challenges in removing the oil. There has been little published research on oil recovery from corn fermentation co-products despite strong economic reasons to remove oil from DDGS, because such oil is partially responsible for the amount of DDGS that can be fed to swine and poultry, but it can be used for biofuel.

The CCDS is a stabilized matrix because of the mixing, shearing, cooking and heating during the ethanol production process. 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 protein, free fatty acids, mono-and di-glycerides, and phospholipids; 2) as oil attached to the surface of hydrophobic protein such as zein and also to carbohydrate material or cell debris; 3) as intact oil bodies in large endosperm and germ particles having 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 (oleosomes, sphereosomes or oil bodies) with alkaline proteins (oleosins) on the surface

(Tzen and Huang, 1992; Young and Schadel, 1990). The lipid bodies contain oil in the core and are surrounded by a half-unit membrane consisting of a phospholipids monolayer and oleosins interacting with the phospholipids and covering the phospholipids layer, thereby stabilizing the oil bodies inside the cells (Huang, 1996; Murphy, 1993). Isolated oil bodies from corn consists of 95% triacylglycerol, 4% diacylglycerol, 0.9% phospholipids, and 1.4% protein (mostly oleosins) (Tzen and Huang, 1992). To release the oil, such oleosomes have to be broken mechanically or chemically.

The cell wall of the corn kernel contains hemicelluloses, celluloses, but no pectin (Karvolic et al., 1994). Therefore, commercial enzymes containing hemicellulases and cellulases should be effective as an enzyme treatment for oil body extraction from CCDS especially from the intact cells. Then a protease treatment should release the free oil. Tzen and Huang (1992) hydrolyzed maize oil bodies with trypsin and oil body membrane broke due to hydrolysis of oleosins. Coalescence was evident among the oil bodies, so such hydrolysis resulted in oil that could be easily separated from the aqueous medium by centrifugation. 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. These particles mostly likely have intact cellular structure, so the use of protease alone may not be effective in releasing the oil. If protease is used in combination with cellulases and hemicellulases, the proteases may be able to gain access to the proteins encapsulating the oil and release of the oil.

In CCDS, the dispersed proteins, unhydrolyzed or residual starch, and broken cell walls may interact with the free oil to stabilize it (Rosenthal et al., 1996). Proteases can hydrolyze the proteins that stabilize the emulsion, hemicellulases, cellulases, xylanases and glucanases may further break down the fragmented cell wall components which may 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 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 to determine how centrifugal force affects oil recovery from CCDS.

Materials and Methods

CCDS was obtained from LincolnWay Energy, a typical ethanol plant in Nevada, IA**.** It was stored in the 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 acid hydrolysis method and used to calculate oil recovery. Zein was obtained from Freeman Industries LLC (Tuckahoe, NY). This industrial-grade zein contained 15.3% nitrogen on a dry weight basis.

Chemicals: Hexanes, petroleum ether and ethyl ether were obtained from Fischer Scientific (Fairlawn, NJ), absolute ethanol was obtained from Underwriters Laboratories (Northbrook, IL). 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). All enzymes were in liquid form and Genencor® enzymes were commercial preparations. They were stored at 4 °C until used. 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 declared activity of 1000 SAPU/g enzyme minimum. One SAPU (Spectrophotometer Acid Protease Units) is the amount that liberates one micromole of tyrosine/min from a casein substrate. The optimum pH of the enzyme was 3 and temperature was 50 °C.

2) Alkaline protease: Alcalase (2.4L type FG), a serine protease extracted from *Bacillus Licheniformis*. The enzyme activity is 2.4 Anison 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 3200 IU/g (minimum). Suggested optimum activity was 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 minimum. 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 (minimum) 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 pectinase units/g. The optimum temperature of enzyme is 45 °C and optimum pH is 3.85.

Compositional analysis of CCDS: The moisture content was determined by using the drying oven at 50 °C until constant moisture content was obtained. Since CCDS caramelizes at higher temperatures, 50 °C was chosen as ideal for the moisture determination. The combustion method was used for determining protein contents using the VarioMax Carbon Nitrogen analyzer (Elementar Analysensysteme Hanau, Germany) (AOAC Official Methods of Analysis, 990.03). Total oil content was determined by acid hydrolysis (AOAC Official Methods of Analysis, 922.06). 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 at 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., 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 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 extraction 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 degrees fixed angle, 7.6 cm radius at 10,000 rpm (8,500 x *g*) for 10 min in 50-mL centrifuge tubes. The separated oil was transferred using hexane at least five times (10-mL each time). The hexane and oil mixture was transferred to preweighed round-bottomed flask. Removal of solvent was done by a rotavapor evaporation system equipped with a heating bath at 60 °C (Buchi rotavapor

R124 and waterbath B-481, New Castle, DE). 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: Three methods were used to reduce the particle size of CCDS. The first method was 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 watts. The power used was 390 watts and treatment time was 10 min. 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 was by grinding, CCDS was placed in a stack of three sieves with sieve openings of 53 µm (U.S. mesh 270), 106 µm (U.S. mesh 140) and 435 µm (U.S. 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 CCDS particles. 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 for 30 min at 10 min intervals. 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, U.K.) 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 distribution analysis**.**

Interaction of oil with hydrophobic protein (zein): Since the protein content in CCDS was about 14% (dry weight basis), 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 the 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 just dispersing zein in water. Zein dispersion was mixed with 20% w/w oil (based on protein or solids content) in a blender and the mixture was blended for 30 min.

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, 5% v/w enzyme concentration at pH 9, 50 °C for 3 h. The control was performed at the incubation conditions of the enzyme-treated samples but without enzyme addition. A heating treatment was conducted at 100 °C for 30 min to determine the effect of heat on hydrophobic interactions. Oil separation and quantification were done 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 x *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 Alcalase 2.4L alkaline protease, Multifect® CX GC cellulase, and Multifect® AA 21 L α-amylase 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 0.1M cacodylate buffer at pH 7.2, 4 °C for 48 h. The primary fixed samples were rinsed twice in 0.1M cacodylate buffer at pH 7.2 and then secondary fixed with 1% osmium tetroxide in 0.1M 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). The resin blocks were polymerized for 48 h at 65 °C. Thick and ultrathin sections of the samples were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Thick sections were contrast stained using 1% toluidine blue. Ultrathin sections 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 200kV using a Gatan Ultrascan 1000 digital camera (Gatan Inc., Warrendale, PA).

Statistical analysis

Statistical analysis to determine significant difference among the different treatments was performed using the statistical analysis software SAS 9.1 (Cary, NC), and one-way Analysis of Variance (ANOVA). 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 the CCDS for the 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. Oil recovery was calculated based on the oil content determined by acid hydrolysis for the specific batch of CCDS used.

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, oil-in-water emulsion stabilized by protein and polar lipids and oil in the oil bodies in intact cells or oil bodies in 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 significantly increased, from 65 to 68%. At high enzyme concentration (20%), the oil recovery was slightly increased to 70%, however, it was not significant above 10% enzyme. The acid protease gave 5% higher oil recovery than the no-enzyme treatments suggesting that there might be hydrolysis of proteins on the free oil bodies subsequently leading to release of oil, and/or destabilization of the oil-in-water emulsion. The remaining 30% oil that could not be recovered may be trapped by polysaccharides or present in the oil bodies of the large endosperm and germ pieces.

The cellulase, Multifect® CX GC did not significantly increase oil recovery at 1% enzyme concentration compared to no-enzyme treatment, 62% vs. 60%. Oil recovery increased slightly at 5% enzyme concentration to 64%, but further increase of enzyme up to 20% did not result in higher oil recoveries. Since cellulases are effective in breaking down the cell wall polysaccharides and should facilitate oil body release (Rosenthal et al., 1996), oil may not have been freed without protease treatment. This cellulase enzyme 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 used, pH 3 vs. pH 4 with pH 3 giving higher oil recovery.

The enzyme dosage used in this experiment was very high because we wanted to examine the maximal potential for oil recovery. The reduction in oil recovery with high concentration of cellulase treatment may have been due to the additional protein added to the system.

During the dry-grind ethanol production, the corn is ground, hydrolyzed, and fermented, releasing much of the oil and oil bodies from the corn (Rosenthal et al., 1996). 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 body membrane, which protect and maintain the integrity of the oil bodies (Jacks et al., 1990). 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 (Tzen and Huang, 1992).

The cell wall of corn kernel is comprised of hemicelluloses, celluloses but no pectin (Karvolic et al., 1994), 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 (Rosenthal et al., 1996). However, Moreau et al. (2004) observed that increasing levels of three cellulases from *Trichoderma reesei* in the hydrolysis of corn germ cell wall components resulted in no obvious trend for increased oil recovery, suggesting that it may be necessary to use cellulases in combination with proteases to significantly increase oil recovery. In aqueous oil extraction, enzymes have been used to increase oil yield by breaking the cell wall and membranes and by hydrolyzing the emulsifying proteins (Moreau et al., 2004). Various enzyme assisted aqueous oil extraction processes have been investigated for canola seeds (Latif et al., 2008), soybeans (Nobrega de Moura et al., 2008; Nobrega de Moura and Johnson, 2009), corn germ (Moreau et al., 2004), and oleosomes from soybeans (Kapchie et al., 2008). Proteases alone or in combination with cellulases significantly improved oil recoveries from flaked soybeans (Lamsal et al., 2006). In our further experiments, the combination of enzymes was used.

Effect of phospholipases on oil recovery: The oil recovery after hydrolyzing with phospholipases is shown in Table 3. G-ZYME® G999 Lyso-phospholipase hydrolyzes the ester bond on sn-1 position of the 1-acylglycerolphosphatide 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 did not significantly increase oil recovery when compared with no-enzyme treatment, 76.4 vs. 76.2%. The phospholipases were expected to hydrolyze the phospholipids half membrane of oil bodies and also the phospholipids stabilized emulsion. Tzen and Huang (1992) reported that hydrolysis with phospholipase A2 and C did not result in hydrolysis of the phospholipids present on the surface of the oil bodies. Huang (1996) suggested that oleosins form a mushroom-like covering on oil bodies, making it inaccessible due to steric hinderance for phospholipases to hydrolyze the phospholipids, and this probably contributed to no increase in oil recovery in this experiment. Phospholipids stabilized the 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 (Rosenthal et al., 1996). During solvent extraction of oil from oilseeds the seed is cracked and flaked to break and rupture the cells for oil extraction (Johnson, 2008). The critical step in aqueous oil extraction processes is grinding because it determines oil yield (Rosenthal et al., 1996). Particle size reduction enhances the enzyme diffusion rates so that the enzymes can easily act on the substrates (Rosenthal et al., 1996).

Three treatments were used to break the CCDS into finer particles in order to increase the surface area for enzyme hydrolysis and to improve oil recovery. The distribution of the particle size after sonication, blending and grinding with mortar and pestle were shown in Figure 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 the blended sample had a 4.4 µm peak size. The sample prepared by mortar and pestle contained sand, so the CCDS particle size could not be accurately measured. The means used for particle size reduction was effective as indicated by these peak particle sizes and the distribution profiles as shown in Figure 1.

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

The oil recovery after blending the CCDS is shown in Table 4. Three different enzymes were used together for the enzyme hydrolysis: Multifect® CX GC cellulase, Multifect® AA 21L α-amylase and Alcalase 2.4L alkaline protease. Previously Multifect® CX GC cellulase when used alone did not significantly increase oil recovery, so it was used in combination with an alkaline protease and amylase. Alkaline protease was used because it is a more pure enzyme compared to the acid protease which is a commercial enzyme preparation, and therefore was expected to be a more effective protease. The α-amylase was used because even after fermentation of the corn, residual starch may remain due to poor conversions and incomplete fermentation (Maisch, 2003) and such starch may form hydrophobic interactions with the lipids.

Blending CCDS resulted in lower oil recoveries even after enzyme hydrolysis compared to samples without blending. More stable emulsions were produced due to severe mixing. The percentage oil recovery after hydrolyzing with a combination of enzyme was 65% (without blending) and 49% (with blending). The greatest percentage oil recovery was obtained after hydrolyzing with alkaline protease alone, 68% (without blending) compared to

59% (with blending). The no-enzyme treatments (controls) gave lower oil recoveries for both the blended CCDS and the non-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 treatment 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 treatment conditions of the two set of 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 changes in pH from 4 to 9 may have contributed to the very low oil recoveries observed. The no-enzyme and blending treatment (incubated 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 show the enzyme treatment was effective in increasing oil recovery.

Oil recovery increased 128 and 45% for the blended CCDS subjected to enzyme hydrolysis with the enzyme combination and alkaline protease respectively, compared to no-enzyme treatments. 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 greatly increased enzyme efficiency.

When CCDS was ground using a mortar and pestle to decrease the particle size of the large CCDS particles, oil recovery significantly increased for the non-enzyme treated ground samples, 82 vs. 78% (Table 5). When enzymes hydrolysis was performed using the combination of Multifect® Pectinase FE and Protex™13FL acid protease, oil recovery did not

significantly increase, 83 vs. 82% when comparing to the 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 centrifuging alone. Since about 20% oil could not be recovered, it suggests that the 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 as evidenced by only 10% oil recovery with centrifugation alone as shown in Table 6. 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 protein was targeted for hydrolysis using the same enzyme in the CCDS, the oil recovery was not as high as in this model, at most 70%. Therefore, freed oil may be attached to other surfaces or may be present as minute droplets.

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 (Lawton and Wilson, 2003). The major storage protein of the corn endosperm is zein, constituting up to 79% (Lawton and Wilson, 2003). 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. 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 granular appearance in 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 20% of the oil that 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 walls, 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 walls. Interestingly the lipid droplets could not be recovered by centrifugation. Blending reduced the 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.

The interaction of the lipid droplets with the protein in the cell wall materials was also viewed using the light microscopy to give a global view as shown in Figure 3. The lipid droplets were visible as dark spheres dispersed throughout the cell and interacting with the protein (Figure 3a), blended CCDS exhibited lipid droplets trapped in the protein matrix (Figure 3b) and CCDS residue after enzyme hydrolysis exhibited the disappearance of the dense protein network and free lipid droplets inside the cell surrounded by degraded protein (Figure 3c).

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. Multifect® Pectinase FE pectinase and Protex™ 13FL acid protease were used together for the enzyme hydrolysis before the centrifuging. As shown in Table 7, oil recovery was not significantly affected by increased centrifugal force, indicating that for the 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 would not further increase oil recovery.

For the non-enzyme treated samples, the CCDS was subjected to the same incubation conditions as the enzyme treatments. There was a slight increase in oil recovery as the centrifugal force increased, with the greatest oil recovery (81%) being obtained at 12,240 x *g*. We expected oil recovery to increase significantly with increasing centrifugal force because of the breakage of the emulsion. A similar trend was observed for the no-incubation controls, with the greatest oil recovery (78%) achieved at the highest centrifugal force. Therefore, when enzymes are used to enhance oil recovery from CCDS, increasing centrifugal force will

not likely increase oil recovery. When there is no enzyme treatment, centrifugal force has a slight effect.

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 (Shahidi and Wanasundara, 2008) with the Folch wash (Folch et al., 1957). Therefore our oil recovery may be underestimated if compared with other solvent extraction results.

Conclusions

Increasing the acid protease concentration increased oil recovery, indicating hydrolysis of protein destabilized the oil-in-water emulsion in the CCDS matrix. When acid protease was used in combination with a cellulase, oil recovery was greater compared to protease alone (81 vs. 70%). 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 be contributing significantly to oil and protein interactions thereby stabilizing the oil in the CCDS matrix as evidenced in the zein and oil model system. Increasing centrifugal force did not increase oil recovery significantly.

Acknowledgements

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Table 1. Oil, protein, and moisture contents (%) of CCDS used.

Composition	Batch	Average % ± SD
Oil (dry basis)	1	17.9*
	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 (wet basis)	1	65.9 ± 0.1
	2	68.4 ± 0.1
	3	68.3 ± 0.2

*Analyzed by Eurofins Scientific Inc., Des Moines, IA, by acid hydrolysis method
CCDS- condensed corn distillers solubles
ND- not determined

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

% Enzyme (v/w) dry weight basis	Protex ™ 13 FL acid protease	Multifect® CX GC cellulase
	% Oil recovery ± SD	
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 within the same column followed by different letters are significantly different ($P< 0.05$).

Table 3. Effects of phospholipases (5%) on oil recovery from CCDS

Treatment	% Oil recovery ± SD
G-ZYME® G999 Lyso-phospholipase + LysoMax™ Microbial phospholipase A2	76.4 ± 0.4a
No enzyme	76.2 ± 0.3a

Enzyme dosage was based on the solids content of CCDS.
Means with different letters are significantly different ($P < 0.05$).

Table 4. Effects of blending and enzyme hydrolysis on oil recovery

Treatment	Blending (Yes/No)	% Oil recovery ± SD
Multifect® CX GC cellulase + Multifect® AA 21 L α-amylase + Alcalase 2.4L alkaline protease (pH 4 and then 9)	No	64.5 ± 1.5ab
	Yes	48.7 ± 8.7bc
Alcalase 2.4L (pH 9)	No	67.9 ± 0.2a
	Yes	59.2 ± 0.2ab
No enzyme (pH 9)	No	59.1 ± 4.3ab
	Yes	40.8 ± 7.6bc
No enzyme (pH 4 and then adjusted to pH 9)	No	51.2 ± 21.5bc
	Yes	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 5. Effects of grinding CCDS on oil recovery

Treatment	Grinding (Yes/No)	% Oil recovery ± SD
Multifect ® Pectinase FE pectinase + Protex™ 13 FL acid protease	Yes	82.6 ± 0.9a
	No	80.7 ± 1.0ab
No enzyme	Yes	82.2 ± 1.4a
	No	78.4 ± 2.4b

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

Table 6. 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 ($P < 0.05$).

Table 7. Effects of enzyme hydrolysis and centrifugation force on oil recovery

Treatment	Centrifugal force (x *g*)	% Oil recovery ± SD
Multifect ® Pectinase FE pectinase + Protex™ 13 FL acid protease	8,500	81.2 ± 1.9ab
	10,280	79.9 ± 1.5bc
	12,240	78.0 ± 0.5c
No enzyme	8,500	77.8 ± 0.8c
	10,280	78.2 ± 0.1c
	12,240	80.9 ± 2.2ab
No incubation pretreatment	8,500	75.8 ± 0.04d
	10,280	77.4 ± 0.1cd
	12,240	78.1 ± 0.4cd

Enzyme dosage (1.5% v/w) based on the solids content of CCDS.
Means followed by different letters are significantly different ($P < 0.05$).

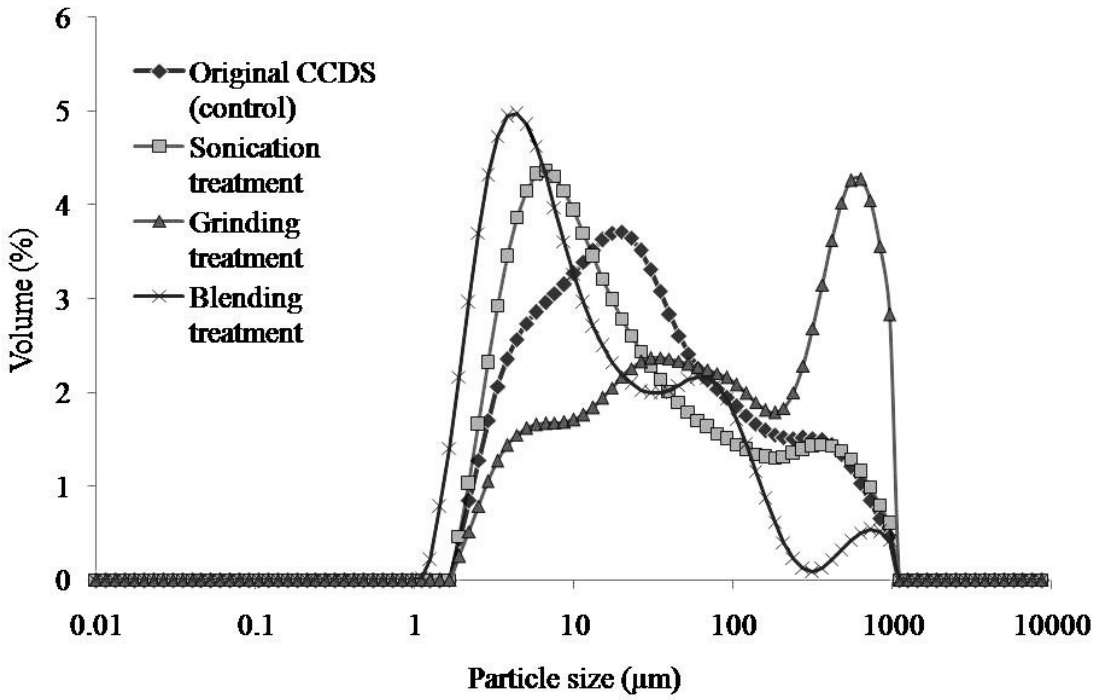
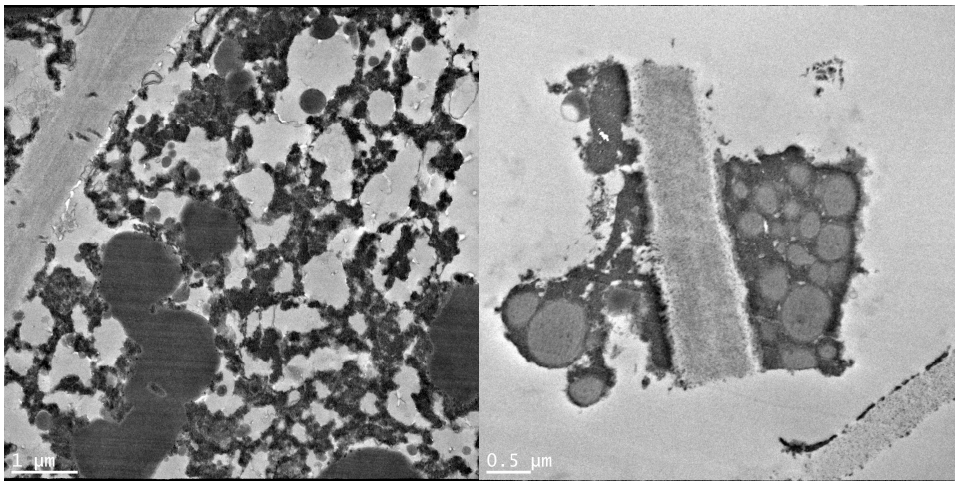
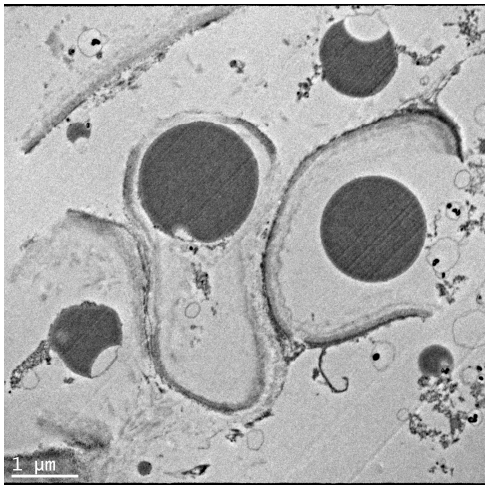


Figure 1. Particle size distribution profile for CCDS samples treated by sonication, blending, and grinding.

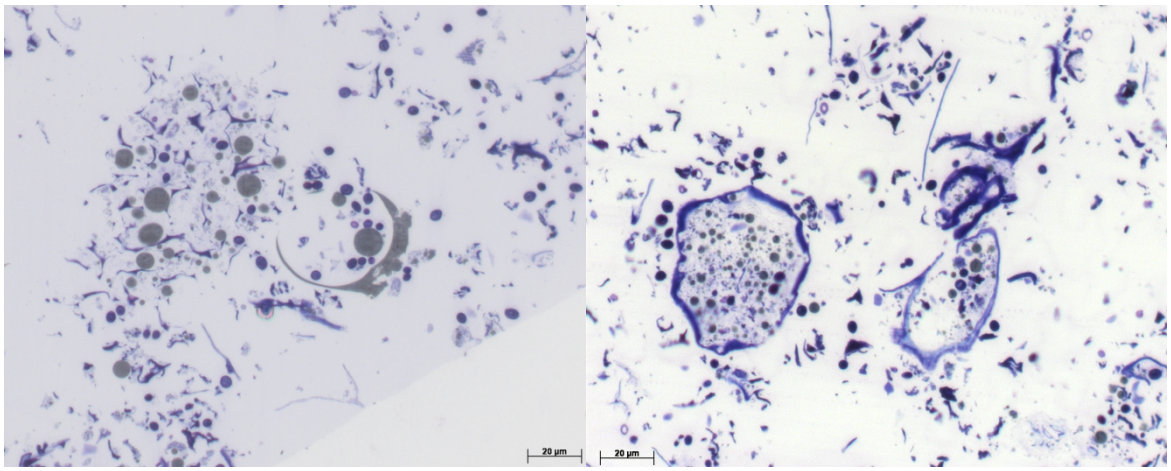


2a 2b

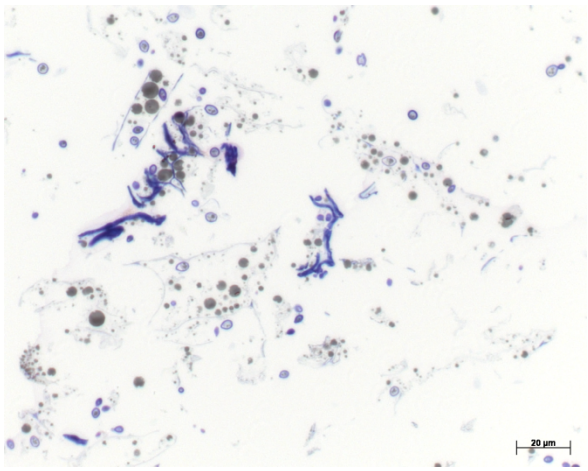


2c

Figure 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 surrounded by degraded protein.



3a **3b**



3c

Figure 3. Light microscopy images of the CCDS**. a**. Original CCDS showing the lipid droplets (dark spheres) dispersed throughout the cell, lipid droplets are shown interacting with the protein. **b**. CCDS after blending showing small lipid droplets trapped in the protein matrix. **c**. CCDS residue after enzyme hydrolysis using cellulase, α-amylase and alkaline protease showing the disappearance of the dense protein network and the presence of free lipid surrounded by degraded protein.

CHAPTER 4. PHYSICAL AND CHEMICAL PROCESSES TO ENHANCE OIL RECOVERY FROM CONDENSED CORN DISTILLERS SOLUBLES

A manuscript submitted to *Bioresource Technology*

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Abstract

Oil recovery from corn fermentation co-products can provide feedstock for biodiesel production. The effects of physical and chemical processes on oil recovery from condensed corn distillers solubles (CCDS) were investigated. Heating provided energy to disrupt physical interactions in the CCDS and increased oil recovery by 2.5-fold when temperature was increased from 25 to 59 °C. Oil recovery at acidic pH was significantly greater than at alkaline pH. Oil recoveries at alkaline pH was increased by heating and addition of the reducing agent, sodium metabisulfite. Oil extraction using polar solvents isopropanol and butanol achieved oil recoveries of greater than 80%. When oil was co-extracted with zein using hexane and ethanol as a co-solvents, the greatest total oil recovery was achieved as 89%. Churning CCDS for 3 h at 50°C, and pH 3.5 achieved up to 80% oil recovery.

Keywords: Corn oil, CCDS, heating, pH, solvent extraction, oil recovery.

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Introduction

The dry-grind corn fermentation process produces dried distillers grains with solubles (DDGS), which is a combination of the condensed corn distillers solubles (CCDS) and the wet distillers grains. About 23 million metric tons of DDGS were produced in 2008 as reported by the Renewable Fuels Association, and this can translate to about 2.76 million metric tons of oil. Assuming 70% of this oil can be recovered, then about 2.2 billion liters (547.6 million gallons) of biodiesel can be made. Oil in feedstock can be a positive energy source, but there are also problems with the high oil content in DDGS, such as it causes softer belly fat in pigs and poor bacon products, and it interferes with normal milk fat production in dairy cattle (Majoni et al., 2009). Therefore, removal of the oil from GGDS or CCDS is expected to improve feed quality and present a significant source for biofuel production.

Several strategies have been explored to recover oil from the dry-grind ethanol co-products. Oil extraction by conventional solvent method from DDGS is not feasible, however, oil recovery by centrifugation of the liquid is generally regarded as a viable method. Normally CCDS contains about 65% moisture, 14% protein, and 20% oil on a dry weight basis, and DDGS contains about 11% moisture, 30-31% protein, and 11-12% oil on a dry weight basis. We are currently developing processing means to have more oil partitioned in the liquid fraction so more oil can be recovered from the CCDS. It is challenging to completely remove the oil from the condensed liquid. We have reported an enzymatic means to improve oil recovery, and this paper describes the physical and chemical means to do so.

CCDS is composed of protein, lipid, fine fiber, and residual starch, and it resembles syrup, so it is also referred to as thick stillage. It is a stable emulsion. The oil may be present in four forms: 1) an oil-in-water emulsion that is stabilized by proteins and phospholipids; 2) oil that is bound to hydrophobic protein, such as corn zeins, and cell wall components; 3) oil present as intact oil bodies of the large endosperm and germ particles; and 4) free but intact oil bodies released from broken germ. There are challenges to recovering oil by centrifugation alone and oil recovery is typically very low.

There have been considerable efforts in developing enzyme-based technologies for extracting oil from oilseeds, but high cost of biocatalysts has slowed the technological acceptance in industry (Gaur et al., 2007). Enzyme-assisted aqueous extraction processes have been used to recover edible oil, eliminating the use of organic solvents and achieving oil recoveries ranging from 53-97% (Rosenthal et al., 1996; Moreau et al., 2007; Nobrega de Moura et al., 2008; Nobrega de Moura and Johnson, 2009). The use of enzymes for extracting oil from CCDS has been reported in our previous paper (Majoni et al., 2009) and they showed some effectiveness when used in combination and with further particle size reduction.

In the present study, we intended to evaluate the use of physical and chemical processes, such as heating, pH changes, high-pressure and temperature, churning, polar solvent extraction, and co-extraction of oil and zein, for oil recovery from CCDS. Heating provides energy required to break emulsion and possibly weaken physical interactions between protein and lipid or carbohydrates and lipid such that oil recovery may be increased (Xu et al., 2007). Increasing or decreasing pH increases the net negative or positive charges on the proteins, therefore affecting protein solubility as an emulsifier (Xu et al., 2007). At

alkaline pH, proteins are easily solubilized, which may make them better emulsifiers (Xu et al., 2007; Bos et al., 1997), or make them release the bound oil. These physical processes, such as changes in pH, temperature, and agitation, may effectively free the oil from its interaction with protein and cell wall materials (Rosenthal et al., 1996, Rosenthal et al., 2001).

Industrial processes for oil extraction from oilseed generally use organic solvents and the solvent of choice is hexanes (Johnson, 1997). Because of environmental and regulatory issues, there is a great interest in developing alternative solvent separation technologies. Isopropanol and n-butanol have higher boiling points than hexane, 82.5 and 117.7 vs. 69 °C, thus the chance of evaporation is lower but energy required for evaporating the solvents is greater (Johnson, 1997). In the present study, isopropanol and butanol were chosen to extract the residual oil from the CCDS residue after the removal of free oil and water by centrifugation, because the solvents can be used for moist plant materials such as wet CCDS. In addition, these solvents can be obtained from renewable resources.

High pressure and temperature treatment may cause extensive hydrolysis of the protein and carbohydrates, therefore, release the oil. Winsness et al. (2007) in a patented high-temperature and pressure cooking for releasing bound oil from whole and thin stillage reported high oil recovery but without detailed quantitative information. Therefore, we evaluated the effect of autoclaving on oil recovery from CCDS.

Sodium metabisulfite is a reducing agent, which breaks or rearranges disulfide bonds between protein subunits or within a peptide chain and makes them more soluble (Xu et al., 2007). The major protein in corn endosperm, zein, has disulfide bonds (Lawton and Wilson, 2003). It is possible that the hydrophobic zein stabilizes the oil-in-water emulsion and may

be destabilized by reducing or rearranging the disulfide bonds and changing its properties. When the protein becomes soluble, oil may be more easily released. Co-extracting zein with oil will result in the removal of zein, disruption of the interations, therefore, improvement of oil extraction.

The objective of the present study was to determine the effects of physical and chemical processes including heating, pH changes, autoclaving, reducing agents, churning, alternative solvent extraction, and co-extraction of zein on oil recovery from CCDS.

Materials and Methods

Condensed corn distillers solubles: CCDS was obtained from LincolnWay Energy, a typical ethanol plant in Nevada, IA, and it was stored in refrigerator at 4°C until used. To prevent mold growth, sodium azide was added to the CCDS. Different batches of CCDS were obtained at different times and used in this study. CCDS, 30% solids content was used unless otherwise stated. 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 acid hydrolysis method and used to calculate oil recovery.

Chemicals*:* Isopropanol, butanol, hexanes, petroleum ether, ethyl ether, sodium metabisulfute, sodium hydroxide, hydrochloric acid were obtained from Fischer Scientific (Fairlawn, NJ). Ethanol was obtained from Underwriters Laboratories (Northbrook, IL).

Enzymes: Enzymes were obtained from Genencor® International Inc. (Rochester, NY) and were liquid commercial preparations. They were stored at 4 °C until used. Multifect® CX GC Genencor® cellulase has cellulase, hemicellulase, xylanase, and glucanase activity. The cellulase is derived from a selected strain of *Trichoderma reesei*. Suggested optimum activity was pH 4 and 55 °C. Experimental soy blend is a Genencor® cellulase. It is a blend of Multifect ® CX B cellulase (42%), Multifect® CX GC cellulase (33%), and Multifect® Pectinase FE (25%). Enzyme specifications were not available for this enzyme blend. Multifect® Pectinase FE is a concentrated liquid pectinase complex from *Aspergillus niger* and contains pectinase, cellulase, and hemicellulase activities. The optimum temperature of enzyme is 45 °C and optimum pH is 3.85. Protex™ 15L is an acid fungal protease obtained from genetically modified selected strain of *Trichoderma reesei* whose optimum pH and temperature is 4.5 and 55 °C respectively.

Determination of composition of CCDS: Moisture content was determined by using a drying oven at 50 °C until constant moisture content was obtained. Since CCDS caramelizes at higher temperatures, 50 °C was chosen as ideal for the moisture determination. The combustion method (AOAC official methods of analysis, method 990.03) and a VarioMax Carbon Nitrogen analyzer (Elementar Analysensysteme, Hanau, Germany) were used for determining protein content. Total oil content was determined by acid hydrolysis (AOAC official method of analysis, method 922.06). The moisture and oil content were measured in duplicate for every new batch of CCDS supplied during the study.

Electron microscopy sample preparation and imaging: CCDS was subjected to transmission electron microscopy imaging to observe the forms of oil's presence. For sample preparation, contents in CCDS were fixed, washed, dehydrated, embedded with epoxy resin as previously described (Majoni et al., 2009). A JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA) was used to capture the images.

Effects of heating treatment on oil recovery: For each of the two replicates, 40 g of CCDS was used. The samples were either subjected to heat treatment at a specified temperature or left at room temperature of 25 °C, incubation time of 10 min. The heat-treated samples were placed in a shaker water bath (Model-R-76, New Brunswick Scientific Co. Inc., NJ) except for the 100 °C treatment samples, which were placed in a beaker containing boiling water. Following heat treatment, oil separation was done using a a Centra MP4 centrifuge (International Equipment Company, Needham Heights, MA) fitted with a 854 rotor, fixed angle of 20 degrees, 7.6 cm radius at 10,000 rpm (8,500 x *g*) for 10 min followed by transferring the separated oil by using hexane (5 times using 10-mL hexane each time). Removal of solvent was done by distillation using a rotavapor evaporation system at 60 °C. Residual solvent was removed using a vacuum oven at 25 °C (National Appliance Company, Portland, OR) for 24 h. The weight of the oil was determined gravimetrically.

Effects of pH and reducing agent on oil recovery: CCDS was adjusted to pH 1, 2, 3, 4, 9, 10, 11, and 12 using aqueous 20% (w/v) sodium hydroxide or 20% (v/v) hydrochloric acid. The pH-adjusted CCDS samples of about 40 g were placed in 50-mL centrifuge tubes. The

treatment conditions, in addition to the different pHs, were ambient temperature (25 °C) as control, use of heating at 100 °C for 60 min, and use of reducing agent (sodium metabisulfite) with heating at 100 °C for 60 min. The concentration of the reducing agent was 1.5% w/w (based on solids content of CCDS). Following the treatments, oil extraction and quantification were performed as previously described.

Oil extraction from the solid residue with polar solvents: CCDS was placed into 250-mL centrifuge bottles (~100g). The treatments were carried out using 91, 81, and 71% v/v isopropanol and butanol for extracting oil from the residue of the CCDS after centrifugation and removal of free oil. The experimental protocol was as follows: the CCDS in 250-mL centrifuge bottles was placed in a water bath at 100 °C to allow heat to destabilize the CCDS matrix. Free oil was separated by centrifugation at 4,000 rpm (2,710 x *g*) for 10 min. The aqueous supernatant was removed, the residue was weighed, and moisture content was measured by using an infrared moisture analyzer (Sartorius MA-30, Elk Grove, IL). The moisture content was used to calculate how much pure solvent was required to make 91, 81, and 71% v/v isopropanol and butanol. Oil was extracted from the residue twice using 450, 184, and 106 mL of isopropanol for the 91, 81, and 71% extraction treatments, respectively. For butanol, 494, 202, and 119 mL of butanol were used for the 91, 81 and 71% treatments, respectively. The incubation time for each extraction was 30 min. For isopropanol, the temperature used for oil extraction was 80 °C, which was 2 °C below the boiling point, and for butanol, the temperature was 90 °C, which was 28 °C below the boiling point.

After each extraction, the mixture was filtered in a sintered glass funnel under vacuum in order to separate the extract from the solid residue. The filtrates were pooled in a

round-bottom flask and concentrated by using a rotary evaporator at 70 °C. Since the solvents extracted non-lipid materials, hexane was used to dissolve and extract lipids from the extracts. The hexane extraction was done three times to ensure that all the oil was extracted. The extracts were placed in a pre-weighed round-bottom flask and the solvent was removed using a rotary evaporator at 60 °C. Residual solvent was removed as previously described and oil was weighed.

Oil and zein co-extraction: The extraction of oil and zein was done in three steps as illustrated in Figure 1. For free oil extraction by centrifugation, CCDS was either subjected to enzyme hydrolysis prior to co-solvent extraction of the residue, or incubated using the same conditions as used for enzyme treatment but with no-enzyme, or left at room temperature for 3 h. For enzyme hydrolysis, Multifect® CX GC Genencor® cellulase and Experimental soy blend Genencor® cellulase at 4% v/w enzyme dosage (based on CCDS dry matter) were used. Incubation was at 50 °C, pH 4 for 3 h. Following incubation, the free oil (oil A) was recovered by centrifuging at 4,000 rpm (2,710 x *g*) for 10 min. Oil transfer and quantification was done as previously described. Residue was separated from the supernatant and moisture content of residue was determined and it was used to determine the amount of pure ethanol needed to give 70% v/v ethanol concentration required for zein extraction. The oil and zein were simultaneously extracted from the residue by using hexane for oil extraction (oil B) and 70% v/v ethanol for zein extraction at 40 °C for 3 h with stirring. The volume of 100% ethanol used is shown in Table 1. For all treatments, 250-mL hexanes was used for oil extraction. Co-extraction was done twice. Following extraction, the mixture was centrifuged in 1 L centrifuge bottles using RC 3B Plus Sorvall® centrifuge with H-6000A swinging

bucket rotor (Kendro Laboratory Products, Newton, CT) at 4,500 rpm (3,493 x *g*) for 10 min. The hexane layer was removed to quantify oil and the ethanol layer was collected for quantifying protein. For zein quantification, the micro-Kjeldahl method was used (AOAC official methods of analysis, 960.52).

Effect of churning treatment on oil recovery: A laboratory stirrer (Eurostar power-b IKA®-Werke lab stirrer IKA®-Works, Wilmington, NC) equipped with a stirring shaft with paddles at a stirring speed 50 rpm was used to mimic butter churning to facilitate the coalescence of free oil droplets in the CCDS. The solids content of CCDS was adjusted to 25% to decrease viscosity and facilitate stirring. Incubation conditions were 50 °C, and pH 3.5 for 3 h and 6 h.

Effects of high temperature and pressure treatment on oil recovery: The CCDS (100 g) was incubated at pH 3.5, and 50 °C for 6 h in a shaker water bath. This was used as the control for the enzyme treatment. Following incubation, the CCDS was autoclaved (Tomy Tech Inc., ES-215/315, Fremont, CA) at 121°C and 103.7 kPa (15.04 psi) for 60 min. For enzyme hydrolysis treatment prior to autoclaving, Multifect® Pectinase FE pectinase and Protex™ 15L acid protease were used at 4% enzyme dosage (based on CCDS dry matter). The enzyme incubation conditions were pH 3.5, at 50 °C for 6 h. Following autoclaving, the CCDS was placed in a water bath at 80 °C to ensure that the same temperature was maintained for all the treatments prior to centrifugation. Controls at various conditions were also used. For oil separation, centrifugation was done at 2,710 x *g* for 10 min as previously described.

Statistical analysis

Statistical analysis to determine significant difference among the different treatments was performed using the statistical analysis software SAS 9.1 (Cary, NC), and one-way Analysis of Variance (ANOVA). 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

Composition of CCDS: The composition of the CCDS for the different batches range from 18-21% for total lipids, 14-19% for protein, and 66-68% for moisture content (Table 2). Oil content and moisture level were the most important parameters, protein content was not as critical. Oil recovery was calculated based on the oil content determined by acid hydrolysis for the batch of CCDS used.

Transmission electron microscopy (TEM) imaging: CCDS was subjected to TEM in order to determine how the oil is associated with other components in CCDS. The lipid droplets are visible as dark spheres and the they are surrounded by dispersed protein (Figure 2). The proteins are seen as a dense network having granular appearance in the cytoplasm. The protein is denatured because of the heat 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 and germ. In our previous research, Majoni et al. (2009) also showed oil attachment to broken cell debris. In addition, if heating destabilizes the CCDS

matrix and free oil is released, it indicates the presence of oil-in-water emulsions. Therefore, all observations confirm the four main forms of oil`s presence in CCDS: 1) oil-in-water emulsion possibly stabilized by proteins and phospholipids; 2) oil bound to hydrophobic protein and cell wall components; 3) intact oil bodies from the large endosperm and germ particles; and 4) free intact oil bodies released from broken germ and corn kernel. Various physical and chemical treatments may have different effects on these forms of oil, and this study was intended to be an observational study. Study of mechanisms of interactions of oil-proteins and oil-carbohydrates can be conducted in future investigations.

Effects of temperature on oil recovery: Increasing temperature increases oil recovery from CCDS as shown in Figure 3. At 25 °C and 42 °C oil recovery was not significantly different. When the temperature was increased to 59 °C, however, oil recovery sharply increased, by approximately 150%. Oil recoveries at 59, 70, 85, and 100 °C were not significantly different. Much of the oil in the CCDS may be in form of oil-in-water emulsion with proteins and phospholipids acting as emulsifiers. A practical means of demulsifying is by heating (Chabrand et al., 2008) as protein denaturation occurs. Thus, a temperature of about 60 °C resulted in the breaking of the CCDS oil-in-water emulsion. The free minute oil droplets attached to hydrophobic surface, free intact oil bodies, or oil bodies in intact cells were not affected by heating and these oils may not be recovered.

Effect of pH on oil recovery: Oil recoveries at acidic pHs (pH 1, 2, 3, and 4) were significantly greater than at alkaline pHs (pH 9, 10, 11 and 12) as shown in Figure 4a. At acidic pHs oil recoveries were not significantly different, with an average of 65%. Oil

recovered at pH 9 was significantly greater than at pH 10, 11 and 12 but lower than at acidic pH. Our results are in agreement with Wu et al. (2009) who recovered less free oil at neutral to alkaline pHs (pH 7 and 8) from cream demulsification. In general, the lower oil recoveries at alkaline pHs suggest that the solubilized proteins may have served as better emulsifiers. We hypothesized that solubilized protein may release the oil better, but this was not the case. The major endosperm protein in corn, zein, has an isoelectric point of 6.2 (Fu et al., 1999), therefore, zein will have lower solubility at pH close to isoelectric point and we expected the uncharged zein to interact with oil even more, giving low oil recovery. These data suggest that acidic pHs are ideal for increasing oil recovery from CCDS. The natural pH of CCDS is about 4.5, which is suitable for oil separation without pH adjustment.

Oil recovery from CCDS at elevated temperature is also dependent on pH. A similar trend was observed (Figure 4b) in which oil recovery at acidic pHs was significantly greater than at alkaline pHs. Oil recovery increased at alkaline pHs compared with the treatments which were not heated. Oil recovery at pH 9 increased from 30 to 56%, indicating that heating facilitated in the breaking of the stabilized oil-in-water emulsion.

Sodium metabisulfite was beneficial in improving oil recovery at alkaline pHs but not helpful at acidic pHs (Figure 4c). The greatest oil recovery without sodium metabisulfite at pH 9 was 56% but when sodium metabisulfite was added, oil recovery increased to 65%. Sodium metabisulfite breaks disulfide linkages between protein subunits or chain segments (Xu et al., 2007), such that protein configuration can be altered and it may have become a less effective emulsifier. Zein protein exists in four forms, α, β, γ, δ, and β, and γ proteins are involved in intra and intermolecular disulfide crosslinking. They contain large amounts of

cysteine residues and require a reducing agent to solubilize them (Lawton and Wilson, 2003). The β and γ zein represent 5 and 20% of the total zein.

The forms of oil which were not significantly affected by pH changes may be free oil bodies and the oil bodies in the large endosperm and germ particles. Therefore, about 15% of oil may be present in such forms.

It is worth noting that oil recovery at 25 °C and pH 4 was higher in this experiment than that in the heating experiment (Figure 3 at 25 °C). It is possible that since the results were derived from different CCDS batches, batch-to-batch variations may exist. Additional research is needed to study batch-to-batch differences and the causes from the ethanol plants.

Effect of alternative solvents on oil recovery: Isopropanol and butanol were the solvents of choice mainly because they are polar and can be used to extract oil from the wet plant materials, such as CCDS. Isopropanol and butanol have lower latent heat of vaporization compared to ethanol, 159.3 and 141.3 vs. 204 cal/g, respectively (Johnson, 1997). Lower latent heat of vaporization suggests less energy is required to vaporize the solvent.

In this experiment, free oil is the oil that can be separated after centrifugation of the CCDS and trapped oil is the oil that remains in the residue/cake of CCDS after centrifugation and that can be extracted by such solvents. The purpose for extracting oil from the residue/cake after free oil extraction was to recover all available oil in CCDS. The solubility of oil in alcohols is dependent on temperature, and oil solubility increases as temperature increases (Johnson, 1997). Feasibility of using isopropanol of 91% v/v for extracting oil from cottonseed was studied in the 1940s (Harris et al., 1947), therefore, this concentration was chosen as the upper limit for both solvents. The other concentrations, 81% v/v and 71% v/v

were chosen for comparison purposes. Lower solvent concentration is more desirable if it can equally extract the oil considering the high moisture content in the CCDS residue.

Oil recovery of the free, trapped and total oil is shown in Table 3. Free oil recovery was not significantly different among all treatments. This was expected since the treatments were subjected to the same experimental conditions. For trapped oil, differences were observed when 71% isopropanol was used, with oil recovery being significantly less (8.3%) than the other treatments. For total oil recovery, there were no significant differences between 71% butanol, and 81 and 91% isopropanol and butanol treatments. Total oil recovery was approximately 85% except for 71% isopropanol, which was 56%. These data suggest that the two-stage oil recovery process is effective as it gave higher oil recovery compared to the previous experiment on effect of pH changes on oil recovery. Butanol is a better solvent because at 71% v/v it can extract as much as 91% v/v butanol. The extraction of oil by solvent is low from unruptured cells, therefore, polar solvents could be used to extract oil from ruptured cells and oil bodies which had the natural compartmentalization destroyed.

Effects of oil and zein co-extraction on oil recovery: The rationale of the co-extraction of oil and zein was that removal of the hydrophobic protein, which is responsible for the strong binding with oil (Majoni et al., 2009), would increase oil recovery. The combined effect of enzyme and solvent was done in order to determine if more oil could be co-extracted with zein from the CCDS residue when enzyme hydrolysis of cellular materials is used. The enzymes were used to breakdown the cell wall components of the CCDS matrix which attach or interact with the oil. Co-extraction of oil and zein resulted in total oil recoveries (free +

trapped oil) of up to 89% as shown in Table 4. When the oil cannot be freed by centrifugation alone, it can be extracted as trapped oil by co-extraction. When such co-extraction was used, enzyme hydrolysis did not seem to be very beneficial. This seems to indicate that the cellulases used did not effectively hydrolyze the intact cells. Majoni et al. (2009) observed slight improvements in oil recovery when cellulase was used alone, and this may be due to the hydrolysis of cell debris and release of oil from the broken cell wall and oil interaction.

The oil recoveries were greater compared to previous treatments suggesting co-extraction of oil and zein can be an effective means of oil recovery. It is likely that when the process is optimized for zein extraction, even higher oil recovery can be reached. More research needs to be done in this direction.

The total protein content in CCDS ranged 14-19% on dry weight basis. The amount of zein extracted relative to the protein content varied depending on whether the sample had been subjected to enzyme hydrolysis. For the enzyme-hydrolyzed samples, the amount of zein recovered relative to total protein content was 14.2 ± 2.5%, whereas for the no-enzyme treatment, the amount of zein recovered was 18.0 ± 0.7% of the total protein present in CCDS. Certain hydrolysis of zein may have occurred by the enzyme treatment, resulting in the low total protein in the extracted zein fraction. It is well known that commercial cellulase preparations have protease activities.

Effect of churning on oil recovery from CCDS: In traditional batch butter churning the oil-in-water emulsion (cream) is inverted to water-in-oil emulsion (Ranjith and Wijewardene, 2006). The cream is destabilized slowly by rotating the churn such that fat globule

membranes are disrupted resulting in fat release. During oil extraction from olives, the crushed olives in water undergo mixing with rotating stainless steel blades at 15-20 rpm in order to break the oil-in-water emulsion and to also facilitate coalescence of the small oil droplets to form larger oil droplets (Petrakis, 2006).

Churning favored the formation of large oil droplets (coalescence) in the CCDS matrix and subsequently increased oil recovery as shown in Table 5. Following 6 h of incubation, oil floated as a layer on the surface of the CCDS and large oil droplets could be seen throughout the CCDS matrix, indicating that stirring allowed the oil droplets to coalesce and float to the top of the CCDS. After 6 h incubation without centrifugation, 47% of the oil could be recovered. With centrifugation, oil recovery was 75%. The 3 h incubation showed oil droplets throughout the CCDS matrix but no separate oil layer and after centrifugation 80% oil was recovered. Therefore, if free oil recovery with no centrifugation is desired, churning can be done for a long period of time. If a centrifuge is available, then less time is needed for churning and high oil recovery can be achieved by centrifugation. There is a great potential for an optimized churning process.

Effects of high temperature and pressure on oil recovery: We hypothesized that autoclaving would hydrolyze CCDS solids (conversion of the suspended solids to dissolved solids).The matrix had a pH of 4.5 which is expected to catalyze hydrolysis of CCDS solids. When comparing oil recoveries, the enzyme-hydrolyzed and autoclaved CCDS had significantly higher oil recovery (74%) compared to other treatments as shown in Table 6. Autoclaving alone gave the lowest oil recovery (62%), and it was not better than the 80°C control. Therefore, autoclave alone was not effective.

Enzyme hydrolysis should have facilitated the breakdown of CCDS cell wall components and proteins that are attached to oil, resulting in oil release. Hydrolysis of proteins on the oil body membrane may have also occurred. However, autoclaving treatment proved to be less effective in improving oil recovery when comparing to other physical and chemical treatments in this study, and the literature result was not supported by our study. Autoclaving may not have resulted in extensive hydrolysis of proteins and fiber in the CCDS matrix to cause release of bound oil.

We should note that a factor contributing to the apparent low oil recovery in our study is the fact that all calculations were based on total oil contained in the CCDS as measured by the acid hydrolysis procedure. This method gives higher total oil values compared to solvent extraction because it results in the total hydrolysis of cellular components. Acid hydrolysis determines both free and bound fat whereas solvent extracts only free fat.

Conclusions

Increasing temperature to about 60 °C increased oil recovery from CCDS since heat can break oil-in-water emulsion. Oil recovery from CCDS was greater at acidic pHs and pH of 3 to 4 was ideal. Use of solvents, such as butanol, may increase oil recoveries up to 85% (free and trapped oil). Churning could be an ideal process for increasing oil recovery from CCDS because after a long incubation period oil can float on top of the CCDS matrix. The oil can be scrapped off without the need for centrifugation. Co-extraction of zein with oil was also effective in improving oil recovery with a major drawback being the labor and cost, however, a major advantage being a co-product zein can be produced. Autoclaving was not an effective means for oil recovery from CCDS.

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Table 1. Ethanol needed for oil and zein co-extraction using hexanes* and ethanol as co-solvents

Treatment	Replicate	Weight of residue/cake (g)	Moisture of residue/cake (%)	Volume of 100 % ethanol used to make 70% v/v ethanol (mL)
CCDS with enzyme at pH 4, 50 °C for 3 h	1	33.03	68.12	52.50
	2	39.20	68.92	63.04
CCDS with no enzyme at pH 4, 50 °C for 3 h	1	49.13	69.47	79.63
	2	51.40	68.36	81.99
CCDS with no enzyme at pH 4, 25 °C for 3 h	1	79.90	67.95	77.83
	2	71.36	67.35	112.14

*For all treatments, 250 mL of hexanes was used as co-solvent.

Table 2. Oil, protein, and moisture contents (%) of CCDS used

Composition	Batch	% Average ± SD
Oil (dry basis)	1	17.9*
	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 (wet basis)	1	65.9 ± 0.1
	2	68.4 ± 0.1
	3	68.3 ± 0.2

*Analyzed by Eurofins Scientific Inc., Des Moines, IA, by acid hydrolysis method.
CCDS- condensed corn distillers solubles
ND- not determined

Table 3. Oil recovery from CCDS by solvent extraction

Solvent concentration	% Free oil	% Trapped oil	% Total (free + trapped oil)
Isopropanol			
71%	51.6 ± 4.9a	8.3 ± 5.3b	56.6 ± 1.1b
81%	56.5 ± 2.7a	56.7 ± 6.2a	82.8 ± 1.3a
91%	53.6 ± 6.4a	67.1 ± 1.6a	85.3 ± 3.3a
Butanol			
71%	51.1 ± 0.5a	59.3 ± 9.2a	81.8 ± 4.5a
81%	52.4 ± 1.9a	60.1 ± 1.5a	83.3 ± 0.4a
91%	50.8 ± 2.4a	67.6 ± 0.3a	84.5 ±1.2a

Means within a column followed by different letters are significantly different ($P<0.05$).

Table 4. Oil recovery from CCDS after co-extraction with zein by hexanes and ethanol

Treatment	% Free oil (A)	% Co-extracted oil (B)	% Total oil (A + B)
Multifect® CX GC + Experimental soy blend	77.0 ± 0.9a	6.8 ± 0.3c	83.8 ± 0.6b
No enzyme	67.7 ± 0.7b	21.3 ± 0.5b	89.0 ± 1.2a
No pretreatment	15.0 ± 2.1c	69.9 ± 3.1a	84.9 ± 0.9b

Means within each column followed by with different letters are significantly different ($P < 0.05$).

Table 5. Effect of churning at 50 °C on oil recovery from CCDS

Treatment	% Oil recovery
3 h incubation	79.7 ± 1.9a
6 h incubation	75.0 ± 1.3b

Means followed by different letters are significantly different ($P < 0.05$).

Table 6. Effect of autoclaving and enzyme on oil recovery from CCDS

Treatment	% Oil recovery
[a]Enzyme hydrolysis + autoclaving	73.9 ± 0.3a
Autoclaving	62.5 ± 0.8c
Control for enzyme + autoclaving	69.9 ± 3.1b
Control at 80 °C	66.1 ± 1.1cb
Control at ambient temperature	67.4 ± 0.2b

[a]Multifect® Pectinase FE and Protex™ 15L acid protease
Means followed by different letters are significantly different ($P < 0.05$).

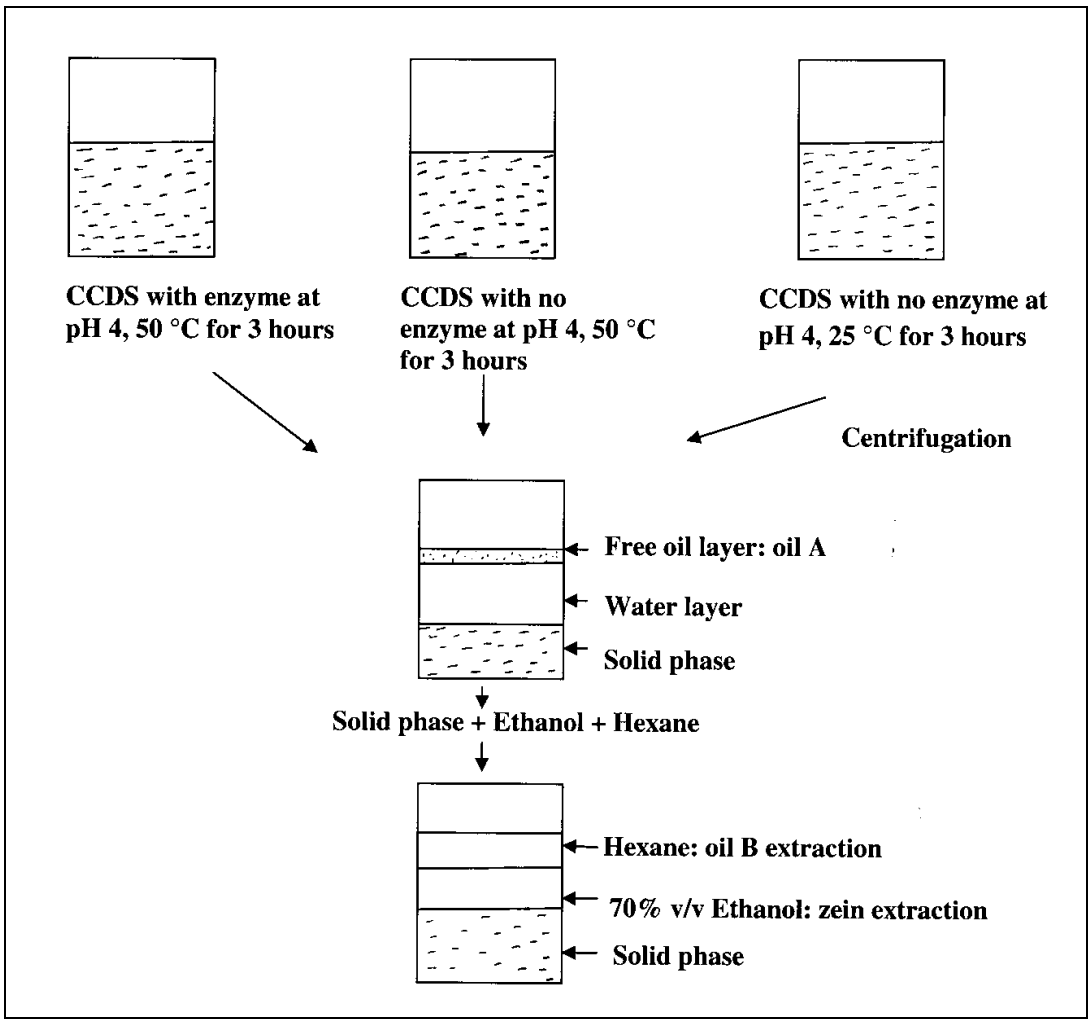


Figure 1. Schematic representation of the oil and zein co-extraction

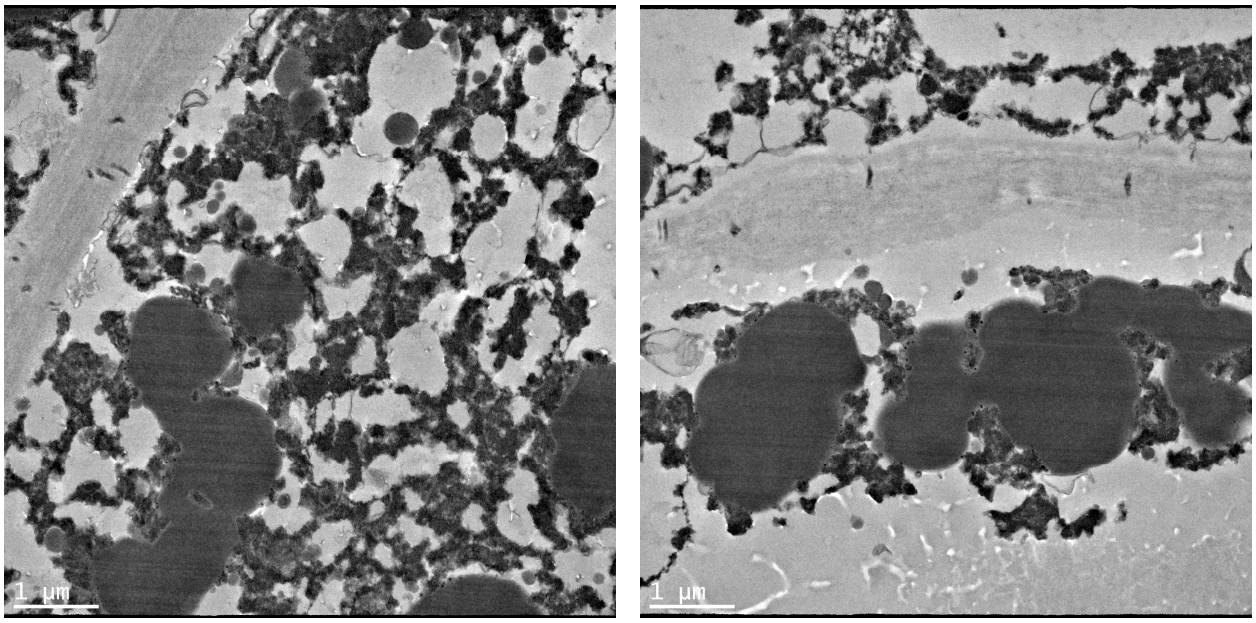


Figure 2. Transmission electron microscopy (TEM) images of the CCDS. The lipid droplets are shown as dark spheres dispersed throughout the cell. The lipid droplets are also shown interacting with the protein. The proteins give a granular appearance to the cytoplasm.

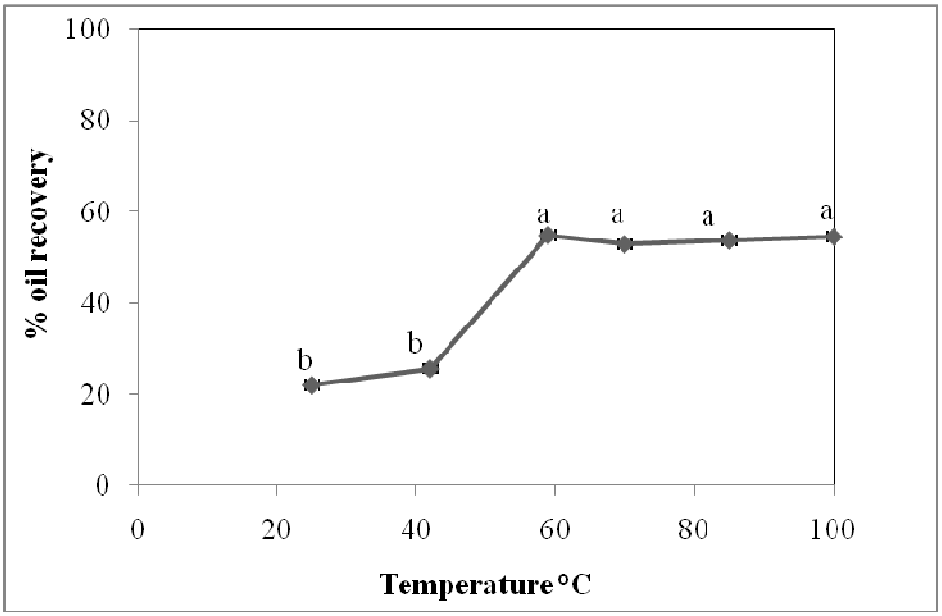
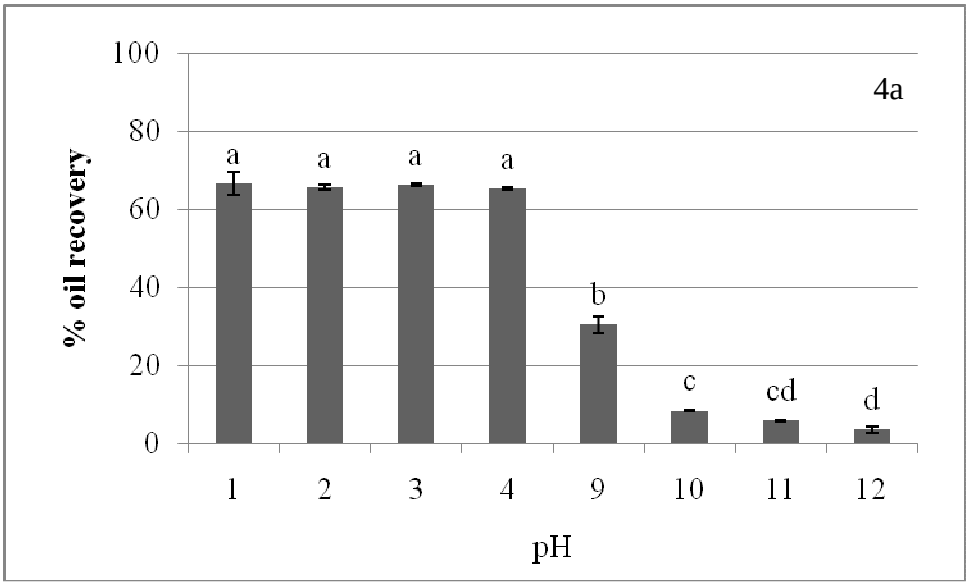
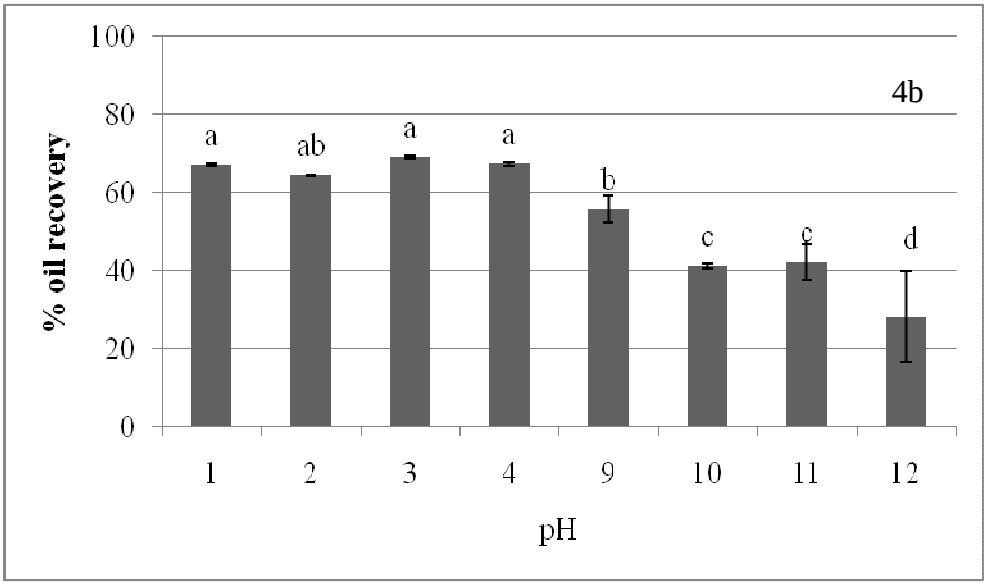


Figure 3. Effect of heating on oil recovery from CCDS. Means followed by different letters are significantly different ($P < 0.05$).





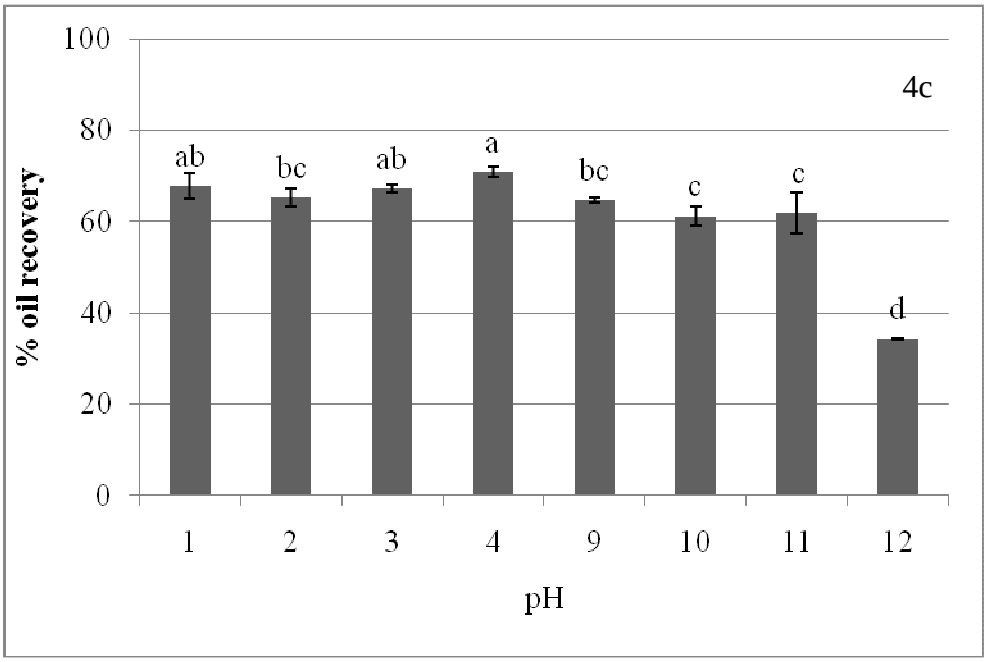


Figure 4. a. Effect of pH at 25 °C on oil recovery from CCDS. **b.** Effect of pH and heating at 100 °C (60 min) on oil recovery from CCDS. **c.** Effect of pH, heating at 100 °C (60 min) and sodium metabisulfite on oil recovery from CCDS. Means followed by different letters are significantly different ($P < 0.05$).

CHAPTER 5. CHARACTERIZATION OF OIL DEPOSIT AND OIL EXTRACTED FROM CONDENSED CORN DISTILLERS SOLUBLES

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Abstract

Oil extracted from condensed corn distillers solubles (CCDS) can form a semi-solid and waxy deposit at the bottom of containers during storage. CCDS is a good source to recover oil, and such oil can be converted to biodiesel. Deposit formation in the extracted oil is mainly a physical stability problem, but it may become a performance problem for biodiesel. The objective of the present work was to determine the composition of the CCDS oil deposit and also determine if valuable phytosterols were present in high concentration. The free fatty acid (FFA) content was very high, 35.7%, and fatty acid composition of the FFA fraction was predominantly palmitic acid, 70.3%. The solid appearance was mainly due to high percentage of high melting free saturated fatty acid. The total unsaponifiable matter was 2.0%, and total phytosterol content was 8.6 mg/g of CCDS oil deposit. Therefore, CCDS oil deposit is a not an enriched source of phytosterols compared to total sterols present in crude corn oil (15.6 mg/g oil). The wax content was high, 2.5 mg/g of CCDS oil deposit compared to 0.5 mg/g of crude corn oil. CCDS oil that is uncentrifugable but polar solvent extractable (trapped oil fraction) was also characterized and found to contain more polar lipids than that in the free oil fraction (centrifugable oil).

Keywords: CCDS oil, CCDS oil deposit, fatty acid composition, wax, phytosterols.

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Introduction

After ethanol fermentation, oil contained in the corn is distributed relatively equally between the stillage and the solid after the centrifugation. The liquid fraction is relatively more oil rich than the solids fraction on the dry weight basis, so oil can be extracted from the liquid for biofuel applications. Corn oil recovered from the corn fermentation co-product condensed corn distillers solubles (CCDS) contains lipids that solidify and settle at ambient (25 °C) temperature. The solidified lipids have been termed “CCDS oil deposit” and it appears sticky and waxy, and is bright orange in color. Such deposit may be a problem when the oil is used to make biodiesel and if the solid fraction cannot be converted to the low melting methyl esters, it may solidify and cause engine failure. Therefore, it is important to characterize the oil deposit and determine why the deposit is semi-solid at room temperatures and to find other potential uses for the deposit. The presence of high-concentration phytosterols in CCDS oil deposit could provide additional revenue for the dry-grind ethanol processing industry.

The oil extracted from co-products of dry-grind corn ethanol production should have the lipid constituents of germ, endosperm, bran, fiber and yeasts (*Saccharomyces cerevisae*). The crude corn oil composition from germ has been reported [1] and also that of whole corn kernel [2], and they are shown in Table 1. Corn kernel contains 3 to 5% total oil [3]. The corn germ contains about 85% of the total oil of the kernel [4] whereas the remainder of the oil is found in the endosperm and hull fractions. The corn germ contains 45 to 50% oil [4].

The yeasts may partially contribute to the fatty acid composition of the corn oil derived from CCDS. The yeast specie *Saccharomyces cerevisae* contains about 9% (db) total lipids [5], and its lipid class composition is also shown in Table 1. The fatty acid composition of this yeast is typically 3% myristic (14:0), 16% palmitic (16:0), 42% palmitoleic (16:1), and 27% oleic (18:1) [5]. However the growth conditions of the yeasts and nutrients have an impact on the fatty acid composition of the yeasts. For example, yeast growing in a medium containing palmitoleic acid (16:1) would result in the 16:1 becoming 91% of the total fatty acid composition and supplementation with oleic acid (18:1) resulted in 18:1 becoming 90% of the total fatty acid [6]. However, for the corn dry-grind ethanol production process, the amount of yeast accumulated at the end of fermentation has not been reported, so the contribution of the yeast lipid to oil content and compositions is unknown.

The composition of the CCDS oil deposit needs to be characterized so potential uses for this oil fraction can be explored, or if the deposit components do not settle and remained in the bulk oil, we would know whether they will affect biodiesel quality or not. Since the CCDS oil deposit separates from the oil at ambient temperature, the presence of elevated level of saturated fatty acids in the deposit is expected. The deposit may also contain a greater proportion of high melting waxes and phytosterols.

CCDS oil may be a good source of phytosterol ferulate esters because ferulate ester is rich in corn fiber oil [7], and during the fermentation of whole corn, the ethanol produced may help solubilize or extract such component from the aleurone layer of the kernel or fiber. Sitostanol ferulate is present in high levels in corn fiber oil [8] and have been found to be very effective in lowering cholesterol in hamsters [9]. Corn fiber oil extracted using hexane gave 3.3% extractable oil of which 4.95 wt % was ferulate esters, 9.1% was phytosterol

esters, 1.0% was free phytosterols [7]. The ferulate esters are similar in structure to those in "gamma oryzanol" found in rice bran oil [10].

In our previous research on oil extraction from CCDS, Majoni et al. [unpublished data] showed that some oil remains trapped in the CCDS solid residue and cannot be separated by centrifugation. The oil is termed trapped oil and characterization of this trapped oil fraction may provide some additional information on why this fraction cannot be separated by centrifugation.

Our research hypothesis is that the CCDS oil deposit contains a high level of saturated fatty acids and high wax content that contributes to the physical appearance at room temperatures. In addition, the CCDS oil deposit may be a good source of phytosterols. The objective of the present study was to determine the composition of the CCDS oil deposit by quantifying the free and total phytosterols, phytosterol ferulate ester, wax content and fatty acid composition. The CCDS oil, free and trapped oil fractions were also characterized.

Material and Methods

All reagents used were of analytical grade. Sitostanol, campestanol were obtained from Supelco (Bellefonte, PA), 5α- cholestane standard was obtained from Sigma-Aldrich (St Louis, MO). A mixture of soy sterols with 95% purity was obtained from Archer Daniels Midlands (Decatur, IL), and it contained β-sitosterol (45.7%), campesterol (27.3%), stigmasterol (15.3%) and brassicasterol (4.4%). The CCDS oil deposit was obtained from LincolnWay Energy (Nevada, IA) and that was collected from the commercially separated CCDS oil. Our own CCDS was obtained from LincolnWay Energy (Nevada, IA) and stored

in the refrigerator at 4 °C until used. To prevent mold growth in CCDS, sodium azide was added.

Free fatty acid content quantification

The free fatty acid content of the corn oil deposit was determined by using the AOCS official method Ca 5a-40 [11]. The CCDS oil deposit (300 g) was washed once or 10 times with hot water (300-mL each time) to remove lactic and acetic acids produced during fermentation that may interfere with the determination of the free fatty acids. These samples were compared to unwashed samples. The percentage of free fatty acids was calculated as oleic acid.

Thin layer chromatography (TLC) separation of the neutral lipids and fatty acid composition determination

To separate the CCDS oil deposit into two lipid fractions of free fatty acid (FFA) and triacylgylcerol (TAG), preparative TLC, 20 x 20 cm, 500 µm thickness Adsorbosil Plus 1 Silica Gel (Alltech Associates Inc., Deerfield, IL) and developing solvent of hexane/diethyl ether/acetic acid (90:10:2 v/v/v) were used. The plate was developed twice to ensure complete separation and then sprayed with 2`,7`dichlorofluorescein and viewed under UV light. Following identification by comparing with standards, the bands were scraped and placed in vials. The lipid fraction were extracted from silica with 10-mL chloroform/methanol (1:1 v/v), three times. The solvent was removed by purging with nitrogen gas.

To produce fatty acid methyl esters (FAMEs) of the lipid fractions, FFA was esterified by using 3% sulfuric acid in methanol for 24 h at 60 °C whereas the TAG fraction was transesterified using 1M sodium methoxide in methanol for 1.5 h at 60 °C. Reactions were terminated with water and FAME was extracted twice using 2-mL of hexane. The CCDS oil deposit, and CCDS oil fatty acid composition were also determined by first transforming the fatty acids into FAMEs with 3% sulfuric acid in methanol for 24 h at 60 °C followed by base catalyzed transesterification with 1M sodium methoxide in methanol for 1.5 h at 60 °C.

The FAMEs were analyzed with the Hewlett-Packard 5890 series II Gas Chromatography (GC), (Avondale, PA) equipped with a flame ionization detector and Supelco™ 2330 capillary column (15 m length x 0.25 mm id x 0.2 µm film thickness) (Bellefonte, PA). Initial oven temperature was 150 °C, oven temperature program was 150-180 °C rate of 5 °C/min, inlet and detector temperatures were 230 °C and the split ratio was 10:1. Sample injection volume was 1 µL.

Total unsaponifiable matter content

The CCDS oil deposit was saponified to free the esterified phytosterols and remove the glycerol lipids. Saponification was done according to the AOCS official method Ca 6b-53 [12]. The saponification was performed for 1 h using 5-mL of 50% KOH, 25-mL 95% ethanol with about 2.5 grams CCDS oil deposit under reflux. The total unsaponifiable extract was saponified again with 5-mL of 50% KOH for 1 h. The procedure was repeated once more to ensure full hydrolysis of the ester bond.

Sample preparation for total phytosterol quantification by GC

The same saponification procedure as described above was used for 2.5 g CCDS oil deposit. The saponification was carried out for 30 min. The unsaponifiable matter was then dissolved in 1-mL of ethyl acetate and streaked on a preparative TLC plate, 20 x 20 cm, 500 µm thickness Adsorbosil Plus 1 Silica Gel (Alltech Associates Inc., Deerfield, IL). The plate was developed using hexane/diethyl ether/acetic acid (90:10:2 v/v/v) and then sprayed with 2`, 7`-dichlorofluorescein and viewed under UV light. The free phytosterol band was collected, 5α-cholestane internal standard was added to the silica, and the silica was extracted with 3 x 10-mL ethanol/diethyl ether/hexane (50:25:25 v/v/v) [13]. The solvent was evaporated under nitrogen and the free sterols were dissolved in ethyl acetate for GC analysis with a FID detector. The free phytosterols were separated on a SAC-5 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Supelco, Bellefonte, PA). The following temperature program was used: 250 °C for 5 min, temperature was then increased to 265 °C at a rate of 1°C/min, and then held at 265 °C for 25 min. The injector and detector temperatures were 280 °C. The flow rate of the carrier gas was 1 mL/min. Phytosterols were identified by comparing the retention times to those of commercial sterols standards. Quantification was carried out by internal standard method.

Ferulate phytosterol separation and GC quantification

Solid-phase extraction (SPE) was performed to separate the phytosterol fraction from neutral lipids. About 0.2 g of the total lipid was dissolved in 2-mL of ethyl acetate and loaded on a 900-mg silica SPE column (Alltech Associates Inc., Deerfield, IL). Neutral lipids were eluted by 15-mL of 5% diethyl ether in hexane [13]. The phytosterols were eluted by a

solvent mixture of 15-mL ethanol/diethyl ether/hexane (50:25:25 v/v/v) [13]. The solvent was evaporated under nitrogen to obtain the phytosterol fraction. Qualitative TLC analysis showed that there was no loss of phytosterols in the neutral lipid fraction and that all the phytosterols were eluted with polar solvent. The phytosterols fraction was redissolved in 0.2-mL ethyl acetate and streaked on preparative TLC plate (20 x 20 cm, 500 µm thickness). The plate was developed using hexane/diethyl ether/acetic acid (60:40:2 v/v/v). Identification of the band was done after spraying with 2`, 7`-dichlorofluorescein and viewing under UV light. Dark blue bands compared to yellow fluorescent sterols above the free phytosterols were identified as phytosterol ferulate ester [13]. The ferulate phytosterol ester band was collected, internal standard added (5α-cholestane) and extracted three times using 10-mL of ethanol/diethyl ether/hexane (50:25:25 v/v/v). The extracts were saponified as previously described. The freed sterols were then quantified by GC as previously described. The total ferulate ester content was calculated based on the free sterols obtained and using the weighted average molecular weight of sterols.

Wax quantification by GC

The CCDS oil deposit was subjected to partial hydrolysis for 30 min in order to obtain the unsaponified wax esters using 2.0 g of the CCDS oil deposit with 5-mL 50% KOH and 25-mL 95% ethanol under reflux conditions (AOCS official method Ca 6b-53) [12]. Qualitative TLC was performed to determine degree of hydrolysis of the glycerol lipids and presence of wax esters. TLC analysis confirmed that the wax esters were present by comparing with standards. Beeswax was used as a control to confirm that partial hydrolysis of the wax esters did not occur under the same saponification reaction.

To separate the wax esters, preparative TLC was performed with hexane/diethyl ether/acetic acid (90:10:2 v/v/v) as developing solvent. The wax ester band was scraped off and extracted three times with 10-mL ethanol/diethyl ether/hexane (50:25:25 v/v/v) [13]. The wax esters were then subjected to alkaline hydrolysis with 2-mL of 50% KOH and 10-mL 95% ethanol for 6 h to fully hydrolyze the ester bond and to produce potassium soap and fatty alcohols. Following saponification of the wax esters, the sample was acidified (about pH 2) with concentrated sulfuric acid to liberate the free fatty acids. The free fatty acids were extracted using 10-mL diethyl ether four times.

The free fatty acids were esterified into FAMEs using 3% (v/v) sulfuric acid in methanol for 24 h at 60 °C after adding the internal standard methyl heptadecanoate (C17:0). Quantification of the FAME by GC was done as previously described, however, oven temperature was held for 25 min at 180 °C for longer chain fatty acids detection. For calculating the wax ester content in the CCDS oil deposit, the wax ester content in the unsaponifiable fraction was calculated using the internal standard method and weighed average molecular weight of fatty acids and fatty alcohols as reported in literature [14]. The wax ester content in the CCDS oil deposit was calculated knowing the total unsaponifiable matter content in the CCDS oil deposit. Since corn kernel wax contains 76% wax esters [14], the wax ester content was then multiplied by 1.32 to give the total wax content of the CCDS oil deposit, so it can be compared to the literature value.

Thermal transition profiles by DSC

The crystallization and melting thermograms of the CCDS oil deposit and corn oil from CCDS were measured using differential scanning calorimetry. The lipid fractions were

transferred to aluminum DSC pans and hermetically sealed. The indium test was used for calibration and the onset temperature as instructed. Nitrogen gas was used as purging gas at flow rate 20.0 mL/min. Sample weight ranged from 3.9 - 10.5 mg. An empty pan was used as reference. The DSC program was 1.0 min hold at -50 °C followed by heating from -50 to 80 °C at 5 °C/min and cooling from 80 to -50 °C at the same rate.

Characterization of neutral and polar lipids of CCDS oil

Free oil from CCDS was obtained by centrifugation of the CCDS using Centra MP4 centrifuge fitted with an 854 rotor, fixed angle 20 degree, 7.6 cm radius at 10,000 rpm (8,500 x *g*) for 10 min in 50-mL centrifuge tubes. The separated oil was transferred using hexane at least five times (10-mL each time). The trapped oil was obtained from the CCDS residue after free oil extraction. It was extracted with chloroform: methanol (2:1 v/v) followed by Folch wash [15]. The solvent and oil mixture was collected and solvent was removed by using the lab scale rotavapor evaporation system at 60 °C. Residual solvent was removed by using a vacuum oven at 25 °C.

Neutral and polar lipid class separation of the free and trapped oils was achieved by solid-phase extraction using a 900-mg silica cartridge (Alltech Associates Inc., Deerfield, IL). Neutral lipid was eluted with 15-mL of chloroform, and polar lipids were eluted sequentially with 5-mL of chloroform: methanol (1:1 v/v), and 10-mL of methanol, and then eluents combined [16]. Solvent was removed by using nitrogen at room temperature and weight of the fractions was recorded.

Qualitative TLC using 20 x 20 cm, 250 µm thickness plates was done on the neutral and polar lipid fractions of the free and trapped oil in order to examine the different lipid

classes present in each fraction. For the neutral lipids, hexane/diethyl ether/acetic acid (90:10:2 v/v/v) was used as developing solvent. For the polar lipids, chloroform/methanol/acetic acid (100:45:5 v/v/v) was used as developing solvent. The plates were then sprayed with 2`, 7`-dichlorofluorescein and viewed under UV light. Identification of the lipid classes was done by using commercial standards.

The polar lipid fraction of the trapped oil was further separated into its major phospholipid classes using preparative TLC with a 20 x 20 cm, 500 µm thickness plate with chloroform/methanol/acetic (100:45:5 v/v/v) as developing solvent. The polar lipid fraction was initially dissolved in 0.25-mL of chloroform, streaked on the TLC plate, sprayed with 2`, 7`-dichlorofluorescein and viewed under UV light. The lipids classes were identified by using commercial standards. The bands of the phospholipid classes were collected and fatty acid determination was carried out by converting the fatty acids into FAMEs with 1M sodium methoxide in methanol for 1.5 h at 60 °C. FAMEs were analyzed by GC as previously described.

Statistical analysis

Statistical analysis to determine significant difference among the different treatments was performed using the statistical analysis software SAS 9.1 (Cary, NC), and one-way Analysis of Variance (ANOVA). 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

Free fatty acid (FFA) content of the CCDS oil deposit

The FFA content of the corn oil deposit is shown in Table 2. Lactic and acetic acids are formed during corn fermentation and they can become dissolved in the oil especially when the FFA content is high [17]. The FFA after washing one time using 1:1 oil: water ratio was not significantly different from washing 10 times, indicating that one wash was sufficient to remove the lactic and acetic acids dissolved in the sample. The unwashed corn oil deposit had a FFA value of 38.3%, which was significantly greater than the washed corn oil deposit. The elevated level of FFA in the corn oil deposit may have partially contributed to the physical state of the oil at room temperature. The presence of a large quantity of FFA allows strong molecular interaction [18] and the tendency of tight molecular packing increases when the FFAs are mostly saturated because they can align themselves better without the double bond “kinks” [18].

Fatty acid composition of FFA and TAG

The fatty acid compositions of the CCDS oil deposit, CCDS oil, FFA and TAG fractions are shown in Table 3. Palmitic acid composition in the TAG fraction and CCDS oil were not significantly different. However, the FFA fraction was characterized by unusually high palmitic acid content (70.3%) compared to that present in the CCDS oil deposit (34.6%), TAG fraction (18.2%) and CCDS oil (13.8%). The fatty acid composition of CCDS oil was similar to that of refined corn germ oil.

The elevated level of palmitic acid may be attributed to the hydrolysis of the ester bonds in TAG sn-positions 1 and 3 by lipase that is specific for sn-1, 3 positions. Positions 1 and 3 tend to be occupied by saturated fatty acids. Since palmitic acid has a high melting point (64-65 °C), it tends to solidify and precipitate under ambient conditions. Therefore, the deposit formed in CCDS oil is enriched in palmitic acid. It should be noted that the deposit sample was collected from a large CCDS oil storage vessel. Therefore, the solidified fraction is highly enriched with saturated FFA. Stearic acid is also enriched in the FFA and TAG fractions. The TAG fraction tends to be more saturated than the CCDS oil.

In addition to the high-melting palmitic acid in the CCDS oil deposit, the high melting phytosterols (138-145° C) [19], waxes (40-120° C) [20] may also be enriched in the CCDS oil deposit.

Total unsaponifiable matter content in the CCDS oil deposit

The total unsaponifiable matter content of the CCDS oil deposit was 2.0% as shown in Table 4. Crude corn oil contains 1.3 to 2.3% of unsaponifiable matter [3], therefore, CCDS oil deposit does not have exceptionally high unsaponifiable matter content. Saponification was carried out three consecutive times for a total of 3 h in order to ensure complete hydrolysis, especially if the sample had high wax content. It was observed that for the 3 consecutive hydrolyses of the CCDS oil deposit, the total unsaponifiable matter did not to change suggesting that 1 h hydrolysis may have been sufficient.

Total phytosterols in the CCDS oil deposit

Quantification of phytosterols in the CCDS oil deposit is shown in Table 5. The total phytosterol content in the CCDS oil deposit was 8.6 mg/g of CCDS oil deposit. Commercially prepared crude corn oil contains about 15.6 mg phytosterols /g oil [21]. Therefore, CCDS oil deposit is a less concentrated source of beneficial phytosterols since it had about 55% of the concentration in crude corn oil. The corn oil deposit contained sitosterol (50.9%) as the most abundant unsaturated phytosterol, followed by campestanol (15.4%), campesterol (7.1%), stigmasterol (5.0%), and sitostanol (3.7%). There was an unidentified component that was quite high in concentration (17.9%). The phytosterol composition was somewhat similar to that of hexane-extracted DDG phytosterols, which contained higher levels of the unsaturated phytosterols, sitosterol (49.6%), campesterol (15.6%) and stigmasterol (5.0%) [22]. The content of saturated phytosterols, campestanol (15.4%) in the CCDS oil deposit was higher than sitostanol (3.7%). The saturated phytosterol composition of the corn oil deposit is similar to that of corn fiber oil, which has relatively high levels of campestanol (10.1%) and sitostanol (8.6%) [13]. These saturated phytostanols are mostly found in corn fiber oil and have been shown to be preferentially esterified with ferulic acid to form ferulate phytosterol esters [22].

Ferulate phytosterol esters are mostly concentrated in the inner pericarp [23]. If the corn kernel is composed of 5-6% pericarp [24] and that the corn fiber (2% extractable oil) is composed primarily of the pericarp, then we can estimate that the concentration of ferulate esters (4 to 5% in corn fiber oil) in crude corn kernel oil will be 0.12 wt % to 0.15 wt %. The ferulate phytosterol content in the CCDS oil deposit was 0.9 mg/g (0.09%) which is less than the estimated ferulate phytosterol content in crude corn kernel oil. Therefore, we conclude

that CCDS oil deposit is a less concentrated source of these beneficial ferulate phytosterol esters.

Wax quantification by GC

The partial hydrolysis of CCDS oil deposit was done for 30 min in order to remove glycerol lipids and leave the wax esters for TLC separation and quantification. Generally wax esters require 2 h hydrolysis time with 5N KOH under reflux for complete saponification [25]. As an example, rice bran wax esters were fully hydrolyzed for 4 h under reflux conditions using 30% KOH in isopropanol [26]. The chemical composition of surface wax of maize inbred WF9 was comprised mainly of 6% alkanes, 2% alcohols, 11% acids, 76% esters and 5% sterols [14] suggesting the maize kernel wax is comprised of mostly wax esters. The composition of the wax esters from maize kernel wax were comprised mainly of 46, 48, 52 and 54 carbon chain length and the predominant esterified fatty acids were C22, C24 whereas the esterified alcohols were C22, C24, C26 and C32 [14]. These data were used in our wax quantification and calculation.

The total wax ester content of the CCDS oil deposit was calculated based on the wax ester content in the unsaponifiable fraction and then converted to the total wax content in the CCDS oil deposit. Since the wax ester content of corn kernel wax is 76%, the wax ester of the CCDS oil deposit was multiplied by 1.32 to give the total wax content. The total wax content in the CCDS oil deposit was 2.5 mg/g as shown in Table 6. The predominant esterified fatty acids were C16 and C18 for the CCDS oil deposit wax ester fraction. Under the experimental conditions for this study, the C22 and C24 esterified acids were not

observed. The wax content in CCDS oil deposit is 5 times greater than that present in crude corn oil (80% greater) which is about 0.5 mg/g.

The presence of high melting wax in the CCDS oil deposit may have partially contributed to the physical appearance of waxiness at room temperature. Melting point of waxes usually ranges from 40 to 120 °C [20].

Neutral lipid phase transitions

The phase transitions temperatures are shown in Table 7. The CCDS oil deposit had higher endothermic peak temperatures than the oil from CCDS. The high melting peak temperatures in the CCDS oil deposit may attribute to the high melting fractions in the oil compared to oil from CCDS. The lower melting phase transitions of the oil from CCDS are consistent with literature [16].

Neutral and polar lipid composition of CCDS oil

The percentage free oil and trapped oil recovered from CCDS was 70 and 30% respectively as shown in Table 8. The total (polar + neutral lipids) of each fraction did not add up to 100% because very polar lipids or non-lipid material may have been present which could not be eluted using the solvents described in our method. The free oil fraction had a significantly greater neutral lipid fraction than that in the trapped oil and the trapped oil had a significantly greater polar lipid fraction than that in the free oil. The fatty acid composition of the phopholipid classes of the trapped oil is shown in Table 9. Phosphatidylinositol (PI) had the most saturated fatty acids, palmitic and stearic compared to phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PE and PC had more unsaturated fatty acids than PI.

Qualitative TLC showed that the neutral lipid fraction of the free oil contained mainly triacylglycerols, free fatty acids, diacylgylcerols, monoacylglycerols, phytosterols and tocopherols. The polar lipid fraction of the trapped oil contained mostly polar lipid classes such as PC, PI, PE, and some TAGs. The presence of high concentration of polar lipids in the trapped oil fraction may explain why this fraction is difficult to extract by centrifugation alone.

Conclusions

The CCDS oil deposit had a high free fatty acid content and very high palmitic acid content compared to CCDS oil. The solid appearance at room temperature was mainly attributed to the presence of saturated fatty acid in the free fatty acid fraction. In addition, the presence of wax at high concentrations may also contribute to the physical characteristics of the CCDS oil deposit. This product is not a rich source for phytosterols. The CCDS oil deposit can be used for making biodiesel and waxes can be removed by winterization. Since the CCDS oil deposit is high in free fatty acid content, acid catalyzed transesterification followed by base catalysis can be used as suggested by Hammond and Wang [27] in making methyl esters. For oil recovery from CCDS, the presence of high concentration polar lipids in the trapped oil fraction may explain why this fraction is difficult to extract by centrifugation alone. Therefore, polar solvent may be used for complete oil extraction, or other physical and chemical means for breaking polar interactions need to be used to improve oil extraction.

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Table 1. Comparison of lipid class composition (%) of corn germ oil, corn kernel oil and yeast lipids

Lipid component	[a]Corn germ oil	[b]Corn kernel oil	[c]Yeast lipids
Triacylglycerol	95.6	75.8	40
Free fatty acids	1.7	1.1	6
Waxes	0.05	-	-
Hydrocarbons and sterol esters	-	3.4	-
Phospholipids	1.5	-	30
Phospholipids and glycolipids	-	13.0	-
Diglycerides and monoglycerides	-	2.1	-
Phytosterol	1.2	4.6	20 (esterified sterols)
Tocopherols	0.06	-	-

[a]Corn germ has 45-50% crude oil on a dry basis [4]
[b]Corn kernel has 3-5% crude oil on a dry basis [3]
[c]Yeasts has 9.0% oil on a dry basis [5]
-Not reported

Table 2. Free fatty acid content of CCDS oil deposit

Sample description	Free fatty acids (%) ± SD
Unwashed	38.3 ± 0.4a
Washed one time	35.7 ± 0.1b
Washed 10 times	36.6 ± 0.4 b

Means followed by different letters are significantly different ($P < 0.05$).

Table 3. Fatty acid composition (%) of the CCDS oil deposit, CCDS oil, free fatty acid (FFA) and triacylglycerol (TAG) fractions

Fatty acid	FFA fraction	TAG fraction	CCDS oil deposit	CCDS oil	* Refined corn germ oil
Palmitic (16:0)	70.3 ± 2.4a	18.2 ± 5.5c	34.4 ± 0.03b	13.8 ± 0.4c	12.2
Stearic (18:0)	5.9 ± 0.1b	16.2 ± 5.8a	4.1 ± 0.0b	2.3 ± 0.07b	2.2
Oleic (18:1)	6.3 ± 0.7b	20.0 ± 5.9a	20.2 ± 0.3a	27.8 ± 0.6a	27.5
Linoleic (18:2)	16.2 ± 1.5c	45.6 ± 5.4b	39.4 ± 0.2b	54.1 ± 0.9a	57.0
Linolenic (18:3)	1.2 ± 0.1b	-	1.7 ± 0.1b	1.9 ± 0.2a	0.9

Means in each row followed by different letters are significantly different ($P< 0.05$).
CCDS- condensed corn distillers solubles
*****Durkee Foods [29]

Table 4. Unsaponifiable matter (%) of the CCDS oil deposit following triple saponifications

CCDS oil deposit	Number of consecutive hydrolysis	Unsaponifiable matter content (%)	Average unsaponifiable matter content (%) after triple hydrolysis ± SD	Overall average (%) ± SD
1	1	2.2	2.1 ± 0.1	2.0 ± 0.1
	2	2.1		
	3	2.0		
2	1	2.1	2.0 ± 0.1	
	2	2.0		
	3	2.0		
3	1	1.9	2.0 ± 0.1	
	2	2.0		
	3	2.0		

Table 5. Total phytosterol contents (mg sterols/g) in CCDS oil deposit

Phytosterol	mg/g unsaponifiable	mg/g of CCDS oil deposit	relative (%)	*average corn germ oil phytosterol (%)
Campestanol	58.9 ± 0.8	1.3 ± 0.0	15.4	-
Campesterol	27.0 ± 5.3	0.6 ± 0.1	7.1	21.4
Stigmasterol	18.9 ± 0.8	0.4 ± 0.0	5.0	6.1
β-Sitosterol	193.9 ± 7.7	4.4 ± 0.3	50.9	69.5
Unknown	68.6 ± 14.4	1.5 ± 0.3	17.9	1.4
Sitostanol	14.2 ± 3.2	0.3 ± 0.1	3.7	-
δ^5-Avenasterol	-	-	-	2.4
Total	381.4 ± 6.4	8.6 ± 0.1	100	-
Ferulate esters	-	0.9 ± 0.3	-	-

-Not reported
*Worthington and Hitchcock [28]

Table 6. Wax content of CCDS oil deposit

Wax ester content in CCDS oil deposit (mg/g)	Total wax content in CCDS oil deposit (mg/g)	Total wax content in commodity crude corn germ oil (mg/g)
1.9 ± 0.4	2.5 ± 0.6	0.5

Table 7. Lipid phase transitions of the CCDS oil deposit and oil from CCDS

Lipid	Heating curve			Cooling curve		
	Onset (T^a)	Peak (T^b)	Terminal (T^c)	Onset (T^a)	Peak (T^b)	Terminal (T^c)
CCDS oil deposit	43.6 ± 4.0	47.6 ± 0.1	49.5 ± 0.5	40.6 ± 0.6	39.1 ±0.7	34.5 ±3.3
CCDS oil	-15.7 ± 6.0	-14.3 ± 4.7	-13.4 ± 4.0	-2.2 ± 14.6	-3.5 ±13.4	-4.8 ± 12.4

[a] Temperature at the start of the transition peak
[b] Temperature of the peak
[c]Temperature of the end of the transition peak

Table 8. Neutral and polar lipid fraction in the free and trapped oil extracted from CCDS

Oil extracted (as % recovered)	Oil (%) ± SD	
	Neutral lipid	Polar lipid
Free oil (70)	88.9 ± 0.4a	1.4 ± 0.1b
Trapped oil (30)	73.2 ± 1.9b	16.3 ± 0.0a

Means in each column followed by different letters are significantly different ($P< 0.05$).

Table 9. Fatty acid composition of the polar lipid fraction of trapped oil

Fatty acid	Fatty acid composition (%)		
	PC	PI	PE
Palmitic (16:0)	19.7	32.6	18.9
Stearic (18:0)	2.9	20.3	6.7
Oleic (18:1)	34.0	1.4	24.5
Linoleic (18:2)	42.4	44.3	48.8
Linolenic (18:3)	0.9	0.3	1.1

Phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE)

CHAPTER 6. GENERAL CONCLUSIONS

Increasing the acid protease dosage up to 10% increased oil recoveries indicating hydrolysis of protein and destabilization of the oil-in-water emulsion in the CCDS matrix. While increasing the cellulase dosage may have resulted in hydrolysis of the cell wall and membrane components, the released oil may have become partially emulsified in the aqueous medium or oil from the released oil bodies cannot be recovered. When the acid protease was used in combination with pectinase, which had cellulase activity, oil recoveries were greater, 81% compared to at most 70% when used alone. CCDS contains large particles from the endosperm and unbroken germ. The ground CCDS showed significant increased oil recovery compared to the unground CCDS for the no enzyme treatments. Particle size reduction by blending may have increased enzyme efficiency but oil recoveries were lower than for unblended CCDS.

The hydrophobic protein zein also contributed to oil and protein interaction thereby stabilizing the oil in the CCDS matrix as evidenced in the zein and oil model system. The presence of oil in the CCDS matrix was shown using transmission electron microscopy. CCDS has intact cell with dispersed protein surrounding lipid droplets inside the cell. In addition, CCDS that had been blended and enzyme hydrolyzed showed oil droplets not attached to protein, suggesting protein hydrolysis by protease. Interestingly the lipid droplets could not be extracted by centrifugation.

Increasing centrifugation force may not increase oil recovery for enzyme treated samples suggesting that the centrifugation force, 8,500 x *g* was sufficient for oil separation in our experiments. Overall, the combination of pectinase and acid protease enzyme preparation

gives higher oil recoveries than using acid protease or cellulase alone. Particle size reduction by grinding of CCDS large particles is also effective in increasing oil recovery from CCDS.

Increasing temperature increased oil recoveries since heat can break oil-in-water emulsions in CCDS. Oil recovery from CCDS was most effectively achieved at acidic pHs and pH of 3-4 would be ideal. Use of solvents such as butanol may increase oil recoveries up to 85% (free and trapped oil) but the techniques may prove to be expensive for the corn ethanol industry. Co-extracting zein with oil was also effective in improving oil recovery with the major drawback being the labor and cost and the major advantage being a co-product zein can be produced.

Churning would be the ideal process for increasing oil recovery from CCDS because after 6 h of incubation at pH 3.5, oil floated on top of the CCDS matrix. The oil can be scrapped off without the need for centrifugation and residual oil can be separated by centrifugation.

Characterization of the CCDS oil deposit derived from CCDS oil showed that the deposit had higher palmitic acid content compared to CCDS oil. The solid appearance at room temperature of the CCDS oil deposit may be attributed to the presence of saturated fatty acid in the free fatty acid fraction and high free fatty content. In addition, the presence of wax at high concentration may also contribute to the physical characteristics of the deposit. The CCDS oil deposit can be used for making biodiesel. Since the CCDS oil deposit has a high free fatty acid content, acid catalyzed transesterification is needed before base catalysis in making methyl esters.

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