A Comparison of Commercial Enzymes for the Aqueous Enzymatic Extraction of Corn Oil from Corn Germ

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ABSTRACT: An aqueous enzymatic method was developed to extract corn oil from corn germ. The basic steps in the method involved "churning" the corn germ with various enzymes and buffer for 4 h at 50°C, and an additional 16 h at 65°C, followed by centrifugation and removal of the oil layer from the surface. No hexane or other organic solvents are used in this process. By using oven-dried corn germ samples (6 g) from a commercial corn wet mill, corn oil yields of about 80% were achieved using three different commercial cellulases. A fourfold scale-up of the method (to 24 g of germ) resulted in oil yields of about 90%. Nine other commercial enzymes were evaluated and resulted in significant but lower oil yields. In the absence of enzymes, oil yields of 27 to 37% were achieved. The chemical compositions of hexane-extracted vs. aqueous enzymatic-extracted corn oils were very similar.

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KEY WORDS: Cellulase, corn, enzymatic, enzymes, extraction, maize, oil, protease, xylanase.

Currently, all commercial corn oil is obtained from corn germ by either hexane extraction (1,2), or a process that combines pressing and hexane extraction (3). Because of the safety and environmental issues associated with the use of hexane, the construction and operational costs of hexane extraction facilities are high. In 2001, the U.S. Environmental Protection Agency issued stricter guidelines for hexane emissions by vegetable oil extraction facilities (4), providing new incentives to develop alternative methods of edible oil extraction. A number of aqueous (5,6), aqueous enzymatic (7–18), and enzyme-assisted solvent extraction (19) methods have been developed, but the current consensus is that hexane extraction is still much less expensive than any of these alternative approaches. Other approaches to replace hexane have focused on using safer solvents such as ethanol (20,21) or vegetable oil itself (22) as extracting solvents.

This project was undertaken to evaluate the possibility of obtaining corn oil from corn germ by applying some of the previously published methods for the aqueous and aqueous enzymatic extraction of oil from oilseeds. Also, since much effort is being devoted to developing less costly enzymes to hydrolyze and ferment cellulosic biomass to ethanol, there will soon be an opportunity to evaluate some of these newly developed enzymes for corn germ extraction. The two key references, which were used as a starting point for this research, were Shi *et al.* (6), who reported an aqueous method that resulted in an 80% yield of peanut oil from peanuts, and Karlovic *et al*. (8), who reported an 80% yield of corn oil from corn germ by using an aqueous enzymatic method with a cellulase.

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MATERIALS AND METHODS

Oven-dried corn germ was generously provided by a wet milling company and upon receipt was stored at 4°C. Enzymes were purchased from Sigma (St. Louis, MO) (Novozyme Alcalase protease P 4860; Novozyme Carezyme cellulase C 2605; Novozyme Celluclast 1.5L cellulase C 2730; Sigma cellulase C 1184; Sigma cellulase C 1794; and Sigma xylanase X 2753), from EMD Biosciences (La Jolla, CA) (Calbiochem Cellulysin cellulase and Calbiochem Macerase pectinase), or were provided by Genencor (Rochester, NY) (Cellulase GC220; Multifect GC cellulase; Multifect xylanase; and Protease GC106).

For hexane extractions, corn germ (6 g) was weighed into a 55-mL glass screw-top tube, and 40 mL of hexane was added. The mixture was homogenized for 1 min at medium speed with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The mixture was shaken horizontally for 1 h at room temperature in a wrist action shaker. Finally, the slurry was filtered through a Whatman Glass Microfiber Filter (GF/A) and evaporated to dryness with N_2.

The procedure for aqueous extraction is summarized in Scheme 1. The procedure for aqueous enzymatic extraction is summarized in Scheme 2. For both procedures, the floating oil layer was removed immediately after centrifugation. Directly below the oil layer was a white interface-emulsion. To remove additional traces of oil from this emulsion, both the oil layer and the upper part of the emulsion layer were removed and placed in a microfuge tube and centrifuged (Schemes 1 and 2). The oil layers from both centrifugations were combined and the total mass was reported.

In the scale-up experiments, 24 g of germ were weighed into a 250-mL centrifuge bottle, and 160 mL of buffer (0.05 M Na acetate, pH 4.0) was added. All other steps were the same as in Scheme 2.

HPLC analysis of the nonpolar lipids was performed in a binary gradient system as previously described (23) with detection *via* an ELSD.

SCHEME 1

Protocol for Aqueous Oil Extraction Studies, Modeled After Shi *et al.* (6)

1. Weigh duplicate 6-g samples of dry germ into 50-mL polycarbonate centrifuge tubes.
2. Add 40 mL buffer, 0.05 M Na acetate, pH 4.0.
3. Grind mixture with a Polytron homogenizer, 2 × 1 min, high speed.
4. Incubate in boiling water bath, 20 min.
5. "Churn" at 65°C for 20 h, with tubes shaking horizontally at 160 rpm in a rotary incubator/shaker.
6. Cool tubes at room temperature for 30–60 min.
7. Centrifuge at 2500 × *g* (4000 rpm) for 60 min in a BHG Hermle Z320 centrifuge.
8. Remove top oil layer with a pipet.
9. Remove the remaining white emulsion-interface (about 1 mL) and centrifuge 10 min at 16,100 × *g* (13,200 rpm) in an Eppendorf microfuge centrifuge 5415 D.
10. Remove additional oil from top of microfuge tube, combine with oil from step 8, and measure mass of total oil.

SCHEME 2

Protocol for Aqueous Enzymatic Oil Extraction Studies, Combining Some of the Features of Shi *et al.* (6) and Karlovic *et al.* (8)

1. Weigh duplicate 6-g samples of dry germ into 50-mL polycarbonate centrifuge tubes.
2. Add 40 mL buffer, 0.05 M Na acetate, pH 4.0.
3. Grind mixture with a Polytron homogenizer, 2 × 1 min, high speed.
4. Add enzyme.
5. "Churn" at 50°C for 4 h, with tubes shaking horizontally at 160 rpm in a rotary incubator/ shaker.
6. "Churn" at 65°C for an additional 16 h, with tubes shaking horizontally at 160 rpm in a rotary incubator/shaker.
7. Cool tubes at room temperature for 30–60 min.
8. Centrifuge at 2500 × *g* (4000 rpm) for 10 min in a BHG Hermle Z320 centrifuge.
9. Remove top oil layer with a pipet.
10. Remove the remaining white emulsion-interface (about 1 mL) and centrifuge for 10 min at 16,100 × *g* (13,200 rpm) in an Eppendorf microfuge centrifuge 5415 D.
11. Remove additional oil from top of microfuge tube, combine with oil from step 9, and measure mass of total oil.

All experimental treatments were performed at least two times, with triplicate samples for each experiment. The values reported are the mean ± SD.

RESULTS AND DISCUSSION

Shi *et al.* (6) recently described a simple aqueous oil extraction method (using no enzymes) that resulted in a yield of about 80% of the oil from peanuts (Scheme 1). When this method was used to extract oil from dried corn germ, the oil yield was about 37% (Table 1), based on the assumption of 100% oil yield from homogenized corn germ extracted with hexane. If the germ was not homogenized prior to hexane extraction, very low oil yields resulted (Table 1).

A second protocol (Scheme 2) was developed to include a 4-h, 50°C enzymatic incubation step, since this temperature is close to optimal for most commercial enzymes. Since buffering the process at pH 4.0 resulted in the aqueous extraction of some oil (37%) from corn germ (Table 1), we decided to continue buffering the process at pH 4.0 when enzymes were added. A pH value of 4.0 is within the useful range for many cellulases and xylanases. Using this protocol, three commercial cellulases (Multifect GC and GC 220 from Genencor and Celluclast from Novozyme) resulted in oil yields of about 80% (Table 2). The boiling step in Scheme 1 was also eliminated because it was repeatedly demonstrated (data not shown) that it had no effect (perhaps because oven-dried corn germ was used and most of its enzymes were probably inactivated during drying). We investigated whether the germ homogenization step could also be eliminated, but because oil yields were reduced by about 10% (Multifect GC, Table 2), the homogenization step was retained. In switching protocols in Schemes 1 and 2, the centrifugation time was shortened to 10 min when it was found that this shorter time was sufficient to float the liberated oil and that additional centrifugation time did not increase oil yields.

TABLE 1

Comparison of the Oil Yields Obtained from Wet-Milled Corn Germ Using Hexane Extraction vs. the Aqueous Oil Extraction Protocol (without enzymes) of Scheme 1

Extraction	Oil yield (wt% oil in germ)	Oil yield (relative %)
Hexane extraction (with homogenization)	42.7 ± 2.0	100
Hexane extraction (without homogenization)	3.6 ± 0.2	7.3 ± 0.4
Aqueous extraction (with homogenization)	15.3 ± 0.4	36.6 ± 1.1

An additional cellulase (Sigma C1794) and two xylanases (Multifect Xylanase from Genencor and Sigma X 2753) produced yields in the range of 54 to 66%. Six other enzymes (including three cellulases, two proteases, and one pectinase) resulted in yields of about 30 to 44%, whereas an oil yield of about 27% was achieved using this protocol (Scheme 2) with no enzyme. Higher "no enzyme" oil yields were obtained using the first protocol (Scheme 1), which included "churning" at 65°C for 20 h, than with the second protocol (Scheme 2), which included a 4-h incubation at 50°C and 16 h of churning at 65°C.

It is interesting to note that six of the seven most effective enzymes in Table 2 are from the fungus *Trichoderma*. Although these enzymes are marketed as cellulases and xylanases, all of them are actually mixtures of enzymes and contain other enzyme activities that may contribute to their ability to extract corn oil. The levels of seven different hydrolytic enzymes (protease, cellulase, β-glucanase, xylanase, hemicellulase, amylase, and the hydrolysis of native starch) were recently compared in a commercial cellulase preparation and a commercial xylanase preparation from *Trichoderma reesei* (24).

The three most effective enzymes from Table 2 were then evaluated for oil yields at several concentrations of each enzyme (Table 3). With each of the enzymes, oil yields reached

TABLE 2
Comparison of Oil Yields Using the Aqueous Enzymatic Oil Extraction Protocol (Scheme 2) with Various Commercial Enzymes, Listed in Order from Highest to Lowest Oil Yields

Enzyme (EC number)	Company[a]	Brand name	Enzyme source	Oil yield[b] (relative %)
Cellulase (EC 3.2.1.4)	Genencor	Multifect GC[c]	*Trichoderma reesei*	81.7 ± 0.7
Cellulase (EC 3.2.1.4)	Genencor	Multifect GC[c] (w/o Polytron homogenization)	*Trichoderma reesei*	70.1 ± 0.6
Cellulase (EC 3.2.1.4)	Novozyme	Celluclast 1.5L[c]	*Trichoderma reesei*	81.5 ± 0.9
Cellulase (EC 3.2.1.4)	Genencor	GC 220[c]	*Trichoderma reesei*	78.8 ± 0.7
Xylanase (EC 3.2.1.8)	Genencor	Multifect Xylanase[c]	*Trichoderma reesei*	65.6 ± 1.4
Cellulase (EC 3.2.1.4)	Sigma	C 1794[d]	*Trichoderma viride*	64.6 ± 3.4
Xylanase (EC 3.2.1.8)	Sigma	X 2753[d]	*Thermomyces lanuginosus*	54.0 ± 0.1
Cellulase (EC 3.2.1.4)	Calbiochem	Cellulysin[d]	*Trichoderma viride*	43.5 ± 2.7
Cellulase (EC 3.2.1.4)	Sigma	C 1184[d]	*Aspergillus niger*	39.6 ± 2.2
Pectinase (EC3.2.1.15)	Calbiochem	Macerase[d]	*Rhizopus*	34.9 ± 1.3
Protease (EC unspecified)	Genencor	GC 106[c]	*Aspergillus niger*	33.3 ± 0.8
Protease (EC 3.2.21.14)	Novozyme	Alcalase[c]	*Bacillus licheniformis*	32.3 ± 1.4
Cellulase (EC 3.2.1.4)	Novozyme	Carezyme[c]	*Aspergillus*	29.9 ± 8.1
No enzyme				27.3 ± 7.3

[a]Genencor, Rochester, NY; Novozyme, Franklinton, NC; Sigma, St. Louis, MO; Calbiochem, La Jolla, CA.
[b]Oil yield relative to hexane extraction (see Table 1).
[c]0.5 mL of liquid enzyme preparation.
[d]50 mg of solid enzyme preparation.

a maximum of about 80% when 0.2 mL was used, and further addition of enzyme did not cause an additional increase in yield.

The two most effective enzymes from Table 2 (Multifect GC and Celluclast) were then compared in a fourfold scale-up experiment (Table 4). Oil yields of about 93 and 91% for Multifect GC and Celluclast, respectively, were achieved in this experiment. We attribute these higher oil yields to the fact that it was easier to recover more of the top oil layer in these larger centrifuge tubes and there was less oil adhering to the walls and associated with the white emulsion layer. It is therefore possible that oil yields of greater than 90% may be achievable by additional scale-ups of the process.

Karlovic *et al.* (8) previously reported an aqueous enzymatic procedure with Celluclast that achieved an 80% yield of corn oil from corn germ. Their procedure also started with corn germ from wet milling, but their corn germ was used while still wet and was not oven dried. Unlike our procedure, theirs also included a "hydrothermal pretreatment" step (pressure cooking at 112°C).

Karlovic *et al.* (8) and Singh *et al.* (25) both noted that, unlike most oilseeds, arabinoxylans are the most abundant carbohydrate polymer in corn germ. Because of this, it is reasonable that enzyme preparations that combine xylanase and cellulase activities may be the most effective (Table 2). Huang (26) demonstrated that lipid bodies, the TAG-containing organelles in seeds, are surrounded by a "half unit" membrane that is composed of a phospholipid monolayer and a structural protein called "oleosin." Therefore, it is reasonable to hypothesize that proteases and/or phospholipases may be useful enzymes for aqueous enzymatic oil extraction. Indeed, Hanmoungjai *et al.* (9) reported that Alcalase (a protease) was useful for the aqueous enzymatic extraction of oil from rice bran. However, the two proteases tested in this study (Table 2) had almost no effect on oil yields. To our knowledge, no one has evaluated phospholipases for their efficacy at enzymatic oil extraction. However, because phospholipases could potentially degrade all cellular biomembranes, and some phospholipases could release lysophospholipids (which are known to act as surfactants), their use may be problematic.

Finally, the chemical compositions of hexane-extracted vs. aqueous enzymatic-extracted corn oils were compared (Table 5). The two compositions were very similar. The very low levels of FFA indicated that lipolytic activity was minimal, even without boiling the germ (indicating that there is very little lipolytic enzyme activity in this oven-dried wet-milled

TABLE 3
Effect of Increasing Levels of Three Cellulases on Oil Yields from the 6-g Germ Procedure (Scheme 2)

Enzyme, brand name (activity units reported by manufacturer)	Volume of enzyme (mL)	Oil yield (relative %)[a]
Multifect GC (82 GCU/g)[b]	0.1	71.2 ± 2.1
	0.2	81.4 ± 0.8
	0.5	82.1 ± 0.3
	1.0	81.2 ± 1.4
Celluclast 1.5L (790 EGU/g)[c]	0.2	79.4 ± 4.1
	0.5	83.5 ± 0.9
	1.0	81.2 ± 2.2
GC 220 (6200 IU/g)[d]	0.1	76.7 ± 1.7
	0.2	80.3 ± 2.0
	0.5	76.7 ± 0.4
	1.0	79.3 ± 1.1

[a]Oil yield relative to hexane extraction (see Table 1).
[b]One GCU is the amount of glucose released from filter paper/h, at 50°C.
[c]One EGU is defined as the amount of enzyme required to reduce the viscosity of a solution of carboxymethylcellulose to one-half at 40°C and pH 6.0.
[d]One IU of activity liberates 1 μmol of reducing sugar/min from carboxymethylcellulose at 50°C and pH 4.8.

TABLE 4
Oil Yields from a Fourfold Scale-up of Aqueous Enzymatic Extraction

Enzyme manufacturer	Oil yield (wt% of germ)	Oil yield (relative %)[a]
Multifect GC	38.2 ± 0.9	93.2 ± 2.2
Celluclast 1.5L	37.3 ± 1.4	91.1 ± 3.5

[a]Oil yield relative to hexane extraction (see Table 1).

TABLE 5
Nonpolar Lipid Composition of Corn Oil Obtained by Hexane Extraction vs. Aqueous Enzymatic Extraction (0.5 mL Multifeet GC) of Oven-Dried Corn Germ

Lipid class	Hexane-extracted oil (wt% of oil)	Aqueous enzyme-extracted oil (wt% of oil)
Sterol fatty acyl esters	0.61 ± 0.01	0.48 ± 0.05
TAG	97.10 ± 3.46	97.95 ± 0.77
Palmitic acid	0.30 ± 0.11	0.10 ± 0.00
Oleic acid	0.13 ± 0.03	0.09 ± 0.01
Linoleic acid	1.09 ± 0.04	0.82 ± 0.07
Free sterols	0.61 ± 0.03	0.24 ± 0.01
Steryl ferulate esters	0.03 ± 0.00	0.00 ± 0.00

corn germ). It is possible that if this aqueous enzymatic method is used with wet corn germ instead of oven-dried corn germ, then boiling may be necessary. The levels of phytosterols (free and esterified) were slightly lower in the aqueous enzyme-extracted oil.

This new aqueous enzymatic extraction process results in oil yields of greater than 90%. This yield is higher than the 80% yield of corn oil from corn germ previously reported by Karlovic *et al*. (8). Care needs to be taken in directly comparing our yields with those previously reported (8) because in the earlier report, wet (undried) corn germ was used as a feedstock for oil extraction while in our present method, factory-dried germ was used. Also, the aqueous enzymatic extraction process used by Karlovic *et al.* (8) included an essential "hydrothermal pretreatment" step, whereas our method resulted in high yields without a hydrothermal pretreatment step. However, if we had used wet corn germ, it is possible that such a step may have been necessary. It should be noted that no precautions were taken to limit the growth of microbes during our aqueous enzymatic process. We recognize that the development of a successful aqueous enzymatic oil extraction process will probably require the implementation of strategies to limit microbial growth. Finally, since several cellulase preparations appear to result in high oil yields, we anticipate that some of the new generation of cellulolytic enzymes that are being developed for biomass hydrolysis and fermentation may result in even higher oil yields and may be more economical to use than the current generation of cellulolytic enzymes.

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