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ORIGINAL PAPER

A Process for the Aqueous Enzymatic Extraction of Corn Oil from Dry Milled Corn Germ and Enzymatic Wet Milled Corn Germ (E-Germ)

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Abstract A bench-scale aqueous enzymatic method was developed to extract corn oil from corn germ from either a commercial corn dry mill or corn germ from a newly-developed experimental enzymatic wet milling process (E-Germ). With both types of germs, no oil was extracted when acidic cellulase was the only enzyme used. Pre-treating dry milled corn germ by heating it in boiling water or microwave pretreatment, followed by enzymatic extraction with the acidic cellulase resulted in oil yields of about 43% and 57%, respectively. A two-step process, combining both acidic cellulase and alkaline protease treatments, with no heat pretreatment, achieved oil yields of 50–65% from dry milled corn germ and 80–90% from E-Germ.

Keywords Corn · *Zea mays* · Oil · Enzymes · Extraction

Introduction

Due to high prices and demand for edible oils for food and non-food (biodiesel) applications, there is an increasing interest in producing corn oil from corn germ produced at new-generation ethanol plants. On-site extraction of oil from germ is desirable since oil is more valuable than germ and more efficient to transport to edible oil refineries. Most corn oil is extracted using a hexane extraction process which requires facilities with high capital and operating costs due to safety and environmental regulations. There is a need for a solvent-free extraction process that could be practiced economically and safely at ethanol plants and corn dry mills so that these smaller facilities could produce another value-added coproduct, corn oil. Previously, we reported a solvent-free aqueous enzymatic oil extraction process that achieved oil yields of 80–90% using corn germ from a commercial corn wet mill [1, 2]. Since our previous report was published new procedures have been reported for the aqueous enzymatic extraction of oil from soybeans [3, 4] and rapeseed [5, 6]. The newly published procedures for both soy and rapeseed involved processes that combined both a cellulase and a protease. In the current study, two commercial proteases were evaluated and one, when incorporated into the previous cellulase-based protocol [1], achieved oil yields of 50–65% from dry milled corn germ and 80–90% from enzymatically wet-milled corn germ (E-Germ) [7].

Materials and Methods

Materials

Dry milled corn germ (~15–20% oil) was obtained from Bunge USA (Danville, IL). In commercial dry milling, the corn kernels are tempered by adding moisture. The kernels are then gently ground in a degerminator, the grits (endosperm fraction) are removed, and the germ and bran are separated by aspiration [8]. Enzymatic wet-milled corn

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germ was obtained from corn kernels (Pioneer 33A14, grown at the University of Illinois Experiment Station during the 2006 season) using the procedure described previously [7]. Briefly, the process for producing E-Germ by enzymatic wet milling of corn kernels involved, first, soaking the corn kernels for 16.5 h at 50 °C in water at a 1:10 mass ratio in tumble dryer. After draining the water, fresh water was added to make a 1:1 mass ratio and the mixture was passed through a coarse mill (Sprout-Bauer) at 1,400 rpm. The pH was adjusted to 4.2 with H_2SO_4 and a thermostable alpha amylase (1.3 mL of Stargen 001 per pound of steeped wet corn) and a protease (0.66 mL GC106 per pound of steeped wet corn) were added. The mixture was incubated at 48 °C for 22.5 h in a tumble dryer. The germ (~40–50% oil, expressed on the basis of germ dry weight) was then skimmed with a comb-like device and spread out in a thin layer to dry overnight on paper. The two cellulases (GC-220 and Multifect GC) and two proteases (GC-106 and Multifect Neutral), were obtained from Genencor. A third protease, Alcalase 2.4L was obtained from Sigma.

Hexane Extraction Controls

For hexane extractions, corn germ (1 g dry milled corn germ or 2 g of E-Germ, dried in an oven for at 55 °C for 24 h) was weighed in 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 (Brinkmann Instruments, Westbury, NY, USA). 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 under N_2. Because there is some batch-to-batch variation in the oil content of both commercial dry milled corn germ and E-Germ, a sample of the germ used in each experiment was also extracted with hexane and this control value was used to calculate the "oil yield relative" for each experiment. Hexane extraction controls were especially important for the E-Germ experiments because the variability in moisture levels of each batch of E-Germ (~45–55%) influenced the accuracy of the oil yield data.

Aqueous Enzymatic Oil Extraction Methods (A Comparison of Schemes 1, 2, and 3)

Three different processes were used to conduct the aqueous enzymatic oil extractions. Scheme 1 included both buffer and cellulase and was identical to the method used in our previous publication which utilized factory-dried corn germ from a commercial corn wet mill (1). Scheme 2 was developed to include new steps to add alkaline buffer and alkaline proteases to the process in Scheme 1. Scheme 3 was developed to replace the need for buffers by simply adjusting the pH with common acid and a common base, to try to make the process more economical and more amenable to scale-up and commercialization.

Scheme 1 (Cellulase Only)

The procedure for aqueous enzymatic extraction with only cellulase is summarized in Scheme 1. For the boiling pretreatment, the dry milled germ (6 g) and buffer (40 mL) were added to a plastic screw-top tube and the tube was heated in a boiling water bath for 30 min. For microwave pre-treatment the dry milled germ (25 g) was placed in a 50-mL glass beaker without buffer and it was subjected to treatment (2 × 20 s, with stirring of germ in between treatments) in a microwave oven (Panasonic 1250 W Household Microwave Oven, Model # NN-5954WF).

Scheme 2 (Cellulase + Protease with Buffers)

The procedure for aqueous enzymatic extraction with cellulase + protease, with buffers, is summarized in Scheme 2.

Scheme 3 (Cellulase + Protease with pH Adjustment but no Buffers were Used)

The procedure for aqueous enzymatic extraction with cellulase + protease, which includes pH adjustment with no buffers, is summarized in Scheme 3.

For all 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, the oil layer and the upper part of the emulsion layer were both removed and placed in a microfuge tube and centrifuged as described in Schemes 1, 2, and 3. The oil layers from both centrifugations were combined and the total mass was reported.

Scale-Up Experiment

In the scale-up experiments, the masses and volumes of all ingredients were 16-fold higher than in the above experiments and the reactions were conducted in a 1-L centrifuge bottle. The first centrifugation was at 2,500×*g* for 30 min at 25 °C in a Sorvall RC-3B Centrifuge (Thermo Fisher Inc. Waltham, MA, USA).

Statistical Analysis

All experimental treatments were performed at least two times, with triplicate samples for each experiment (except

the scale-up study as noted in Table 4). The values reported are the means ± standard deviation. Analysis of variance was conducted to determine statistical significance ($p < 0.05$) by the Bonferroni least significant difference method [9].

Results and Discussion

Experiments with Cellulase Alone (Scheme 1)

In the first experiment no oil was obtained when we used our previously-published AEOE protocol for wet milled corn germ [1] and substituted dry milled corn germ instead of wet milled corn germ (Table 1). After boiling the germ in buffer for 30 min or microwave-pre-treating it without enzyme addition, low yields of oil were observed, 14.3% and 16.8% (not significantly different), respectively. When cellulase (GC-220) was added to the boiled and microwave-pretreated dry milled germ samples, the oil yields were significantly higher (56.6%) for the microwave pretreatment than for the boiling-treatment (42.6%). In a recent report [10], we presented microscopic evidence that microwave pretreatment of corn germ destroys the lipid body membranes and causes the oil in the lipid bodies to coalesce. These microwave-induced ultrastructural changes were thought to cause an increase in oil yields using a screw press [10]. In the current study we believe that these ultrastructural changes were also responsible for the increased oil yields using our aqueous enzymatic oil extraction process (Table 1).

Scheme 1 Protocol for aqueous enzymatic oil extraction of corn oil from wet milled corn germ; previously reported in Moreau et al. [1]

1 Weigh triplicate 6 g samples of corn 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 0.5 mL GC 220 to each tube
5 "Churn" at 50 °C for 4 h, with tubes shaken horizontally 160 rpm in a rotary incubator/ shaker
6 "Churn" at 65 °C for an additional 16 h, with tubes shaken horizontally at 160 rpm in a rotary incubator/shaker
7 Cool tubes at room temperature for 30–60 min
8 Centrifuge at 2,500×*g* (4,000 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 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

Experiments with Cellulase + Protease with Buffers (Scheme 2)

Because others have recently reported that proteases (specifically Multifect Neutral and Alcalase) increased the yield of oil in AEOE processes for soybean [3, 4] and rapeseed [5, 6] a new protocol was designed (Scheme 2) to modify our previous protocol to include these alkaline proteases (Table 2). The addition of Alcalase and Multifect Neutral without cellulase resulted in oil yields of about 52% and 41%, respectively (Table 2). The highest oil yields, 65.6% were achieved with the combination of GC220 and Alcalase. However, the oil yields with the first protease, Alcalase alone was not significantly different than the two treatments that included an acidic cellulase step (Alcalase + GC220 or Alcalase + plus Multifect GC were not significantly different than with Alcalase alone). Similarly, the oil yield with the second protease, Multifect Neutral alone was not significantly different than the oil yields of the treatments with Multifect Neutral + GC220 or Multifect Neutral + plus Multifect GC. A third acidic protease, GC-106, was also evaluated but it resulted in much lower oil yields than Alcalase or Multifect Neutral (data not shown).

Scheme 2 Modified protocol for aqueous enzymatic oil extraction of corn oil from wet milled corn germ, to include the addition of alkaline protease and buffer

1 Weigh triplicate 6 g samples of corn 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 0.5 mL GC220 to each tube
5 "Churn" at 50 °C for 2 h, with tubes shaken horizontally 160 rpm in a rotary incubator/ shaker
6 Add 4.0 mL of 4 M potassium phosphate dibasic to raise pH to ~8.2, shake tubes and then add 0.5 mL alcalase or multifect neutral
7 "Churn" at 50 °C for 2 h
8 "Churn" at 65 °C for an additional 16 h, with tubes shaken horizontally at 160 rpm in a rotary incubator/shaker
9 Cool tubes at room temperature for 30–60 min
10 Centrifuge at 2,500×*g* (4,000 rpm) for 10 min in a BHG Hermle Z320 centrifuge
11 Remove top oil layer with a pipet
12 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
13 Remove additional oil from top of microfuge tube, combine with oil from step 11, and measure mass of total oil

Scheme 3 Modified protocol for the aqueous enzymatic oil extraction of corn oil from dry milled corn germ and E-Term with pH adjustment steps but with no buffers

1. Weigh triplicate 6 g samples of corn germ into 50 mL polycarbonate centrifuge tubes
2. Add 30 mL distilled water
3. Grind mixture with a Polytron homogenizer, 2 × 1 min, high speed
4. Adjust pH to 5.0 (add 1 M H_2SO_4 to decrease pH of dry milled germ and add 1 M KOH to increase pH of E-Germ)
5. Add 0.5 mL GC-220
6. "Churn" at 50 °C for 4 h, with tubes shaken horizontally 160 rpm in a rotary incubator/ shaker
7. Adjust pH to 8.0 or 9.0 with 1 M KOH and add 0.5 mL alcalase 2.4L
8. "Churn" at 50 °C for 2 h
9. "Churn" at 65 °C for an additional 16 h, with tubes shaken horizontally at 160 rpm in a rotary incubator/shaker
10. Cool tubes at room temperature for 30–60 min
11. Centrifuge at 2,500×*g* (4,000 rpm) for 10 min in a BHG Hermle Z320 centrifuge
12. Remove top oil layer with a pipet
13. 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.
14. Remove additional oil from top of microfuge tube, combine with oil from step 13, and measure mass of total oil

Experiments with Cellulase + Protease with pH Adjustment but no Buffers (Scheme 3)

Because our overall goal is to develop a bench scale aqueous enzymatic oil extraction process which can be scaled to the pilot and then industrial level, we decided to try to modify Scheme 2 to remove the use of buffers and instead use only pH adjustment with a common industrial acid (H_2SO_4) and base (KOH). Using this new protocol (Scheme 3) with dry milled corn germ, an oil yield of 64.1% was achieved (Table 3). The new protocol (Scheme 3) was then evaluated using a second source of germ, E-Germ, which was prepared by a new enzymatic wet milling process [7]. When E-Germ was evaluated with the pH 8 adjustment step but without adding Alcalase, no oil was obtained (similar to the results reported for dry milled corn germ in Tables 1 and 2). When E-Germ was evaluated with the pH 8 adjustment step with Alcalase, an oil yield of 77.6% was achieved (Scheme 3) and a slightly higher oil yield (80.9%) was achieved when the pH was adjusted to 9.0 before adding Alcalase, however statistical analysis revealed that these two values were not significantly different. Zhang et al. [5], reported that for the aqueous enzymatic extraction of rapeseed, it was necessary to include an alkaline "extraction" at pH 10.0 to 11.0, before addition of alkaline protease. The authors surmised that this alkaline extraction step was necessary to "solubilize more protein in the aqueous phase and hence enhance the protease hydrolysis and protein extractability." However, with corn germ, the addition of a pH 10.0 treatment step for 30 min, before adjustment to pH 8.0 or 9.0 and before the addition of alkaline protease had no effect on oil yields (data not shown).

Table 1 A comparison of the oil yields obtained from dry milled corn germ using the protocol optimized [1] for wet milled corn germ (Scheme 1)

Germ pretreatment	Enzyme	Oil yield wt% oil in germ	Oil yield relative %[1]
None	None	0[d]	0[d]
	GC220	0[d]	0[d]
Boil germ in buffer (30 min)	None	2.6 ± 0.2[c]	14.3 ± 0.9[c]
	GC220	7.6 ± 0.4[b]	42.6 ± 2.3[b]
Microwave-pretreated dry germ (2 × 20 s)	None	3.0 ± 0.2[c]	16.8 ± 1.1[c]
	GC220	10.2 ± 1.1[a]	56.6 ± 6.3[a]

Means ± standard deviation ($n = 3$)

[1] For each experiment a control sample of germ was extracted with hexane and the aqueous enzymatic oil yields are expressed as relative to hexane extraction (hexane extraction = 100%)

Mean in the same column with no letter in common are significantly different ($p < 0.05$) by the Bonferroni least significant difference method [9]

Scale-Up Experiment

In the final experiment, the two new aqueous enzymatic oil extraction processes (Schemes 2, 3) were scaled up 16-fold (Table 4). When two different samples of E-Germ were evaluated using the scaled up version of Scheme 2, oil yields of about 87% were achieved (Table 4). When the protocol in Scheme 3 was scaled up, slightly lower oil yields were achieved with E-Germ (79.5%) and dry milled corn germ (about 45%), compared to results reported at the smaller scale (Tables 2, 3).

Conclusion

We have developed a new aqueous enzymatic oil extraction process that can achieve oil yields of 50–65% from dry milled corn germ and 80–90% from E-Germ. An advantage of this new process is that it does not include cooking or drying of the germ, thus saving energy costs. Further study is required to try to learn why the oil yields are much higher with E-Germ than with dry milled corn germ. It is possible that the dry milled germ contains other barriers than those in E-Germ and additional enzyme types or higher concentrations may be needed to obtain good oil

Table 2 A comparison of the oil yields obtained from dry milled corn germ using the new protocol modified to include an alkaline protease, with buffers (Scheme 2)

Cellulase	Protease	Oil yield wt% oil in germ	Oil yield relative %[1]
GC220	None	0^{c}	0^{c}
Multifect GC	None	0^{c}	0^{c}
None	Alcalase	9.3 ± 0.7^{ab}	51.8 ± 3.9^{ab}
GC220	Alcalase	11.8 ± 0.5^{a}	65.6 ± 2.5^{a}
Multifect GC	Alcalase	11.4 ± 0.7^{a}	63.6 ± 4.1^{a}
None	Multifect neutral	7.4 ± 1.7^{b}	41.2 ± 9.7^{b}
GC220	Multifect neutral	9.6 ± 0.4^{ab}	53.4 ± 2.1^{ab}
Multifect GC	Multifect neutral	8.6 ± 1.5^{b}	47.9 ± 8.2^{b}

Means ± standard deviation ($n = 3$)

[1] For each experiment a control sample of germ was extracted with hexane and the aqueous enzymatic oil yields are expressed as relative to hexane extraction (hexane extraction = 100%)

Mean in the same column with no letter in common are significantly different ($p < 0.05$) by the Bonferroni least significant difference method [9]

Table 3 A comparison of the oil yields obtained from dry milled corn germ and E-Germ using the new protocol modified to include cellulase and protease with pH adjustment steps, but without buffers (Scheme 3)

Germ	Alcalase added	Buffer pH of protease step	Oil yield wt% oil in germ	Oil yield relative %[1]
Dry milled germ	Yes	8	15.5 ± 1.0^{b}	64.1 ± 4.2^{b}
E-Germ	No	8	0^{c}	0^{c}
	Yes	8	32.9 ± 0.5^{a}	77.6 ± 1.3^{a}
	Yes	9	34.3 ± 1.5^{a}	80.9 ± 3.5^{a}

Means ± standard deviation ($n = 3$)

[1] For each experiment a control sample of germ was extracted with hexane and the aqueous enzymatic oil yields are expressed as relative to hexane extraction (hexane extraction = 100%)

Mean in the same column with no letter in common are significantly different ($p < 0.05$) by the Bonferroni least significant difference method [9]

Table 4 A comparison of the oil yields obtained from E-Germ and dry milled corn germ using the new protocol modified to include an alkaline protease, with buffers (Scheme 2) and without buffers (Scheme 3) and scaled up 16× compared to Tables 2 and 3

Germ	Scheme	Oil yield wt% oil in germ	Oil yield relative %[a]
E-Germ	2	41.0	87.4
	3	33.7	79.5
Dry milled germ	3	8.0	44.6

Means ($n = 2$)

[a] For each experiment a control sample of germ was extracted with hexane and the aqueous enzymatic oil yields are expressed as relative to hexane extraction (hexane extraction = 100%)

yields with dry milled germ. Although the enzymes used in this study are marketed as cellulases and proteases, they are actually complex mixtures of many different types of enzyme activities (including xylanases and many other hydrolases that degrade various carbohydrates, proteins, and lipids), and caution must be exercised to attribute enzymatic oil extraction efficiency to only their cellulase or protease enzyme activities. It is possible that the protease (GC106) used to produce E-Germ may contribute to making the germ a better feedstock for the process than dry milled germ. It is also possible that because the germ is removed in an aqueous environment in the E-Germ process, the germ protein structure may be better preserved, compared to the dry milling, where air and oxygen may alter the structure of the oleosins [2] and other proteins in the germ.

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