Enzymatic degumming of corn oil using phospholipase C from a selected strain of *Pichia pastoris*

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ABSTRACT

Degumming is a necessary refining step for all crude vegetable oils. Purifine™ PLC is a new enzyme used for industrial oil degumming. In the present study, enzymatic degumming trials were performed on crude corn oil using a commercial phospholipase C enzyme Purifine™ PLC with the aim of determining its optimum process conditions. Enzymatic degumming applying 200 mg/kg of Purifine™ PLC during 120 min at 60 °C, and a pH of 5.7 using chemical conditioning, resulted in a residual phosphorus content of 27 mg/kg and an absolute diacylglycerol increase of 0.54 wt%. Compared to water degumming, enzymatic degumming with Purifine™ PLC provided a better degumming (67 mg/kg versus 27 mg/kg) and an increase in the diacylglycerol content. The pH adjustment of crude corn oil performed by means of a caustic pretreatment was not able to keep the pH at an optimal stable value due to the continuous release of acidic phosphate groups. Analysis of the composition of remaining phospholipids in the gums fraction showed that Purifine™ PLC could effectively convert phosphatidylcholine and phosphatidylethanolamine into diacylglycerols, whereas it could not convert phosphatidylinositol and phosphatidic acid. These results confirm that Purifine™ PLC degumming is a commercially feasible alternative to traditional degumming processes.

1. Introduction

Corn oil is well-known for its pleasant flavor, low levels of saturated fatty acids and high levels of polyunsaturated (essential) fatty acids (Moreau, Lampi, & Hicks, 2009). To obtain edible oils from crude oils, a series of refining operations, including water degumming, neutralization, bleaching, and deodorization, are needed (Sampaio et al., 2017; Vaisali, Charanyaa, Belur, & Regupathi, 2015). Water degumming is the first stage of the refining process which removes phospholipids, proteins, and mucilaginous gums. An efficient removal of the phospholipids during oil refining is essential for the production of high quality oils. Residual phospholipids can cause darkening of the oil, result in off-flavors, and impose difficulties on the oil downstream processing (De Greyt & Kellens, 2005; Dijkstra, 2009).

Hydratable phospholipids can be simply removed by water, while an acid treatment is necessary for the removal of the non-hydratable phospholipids. Although acid treatments, such as super degumming (Segers, 1982) and top degumming (Dijkstra & Van Opstal, 1989) are widely used methods, they are not suitable for all types of oils, regardless of their initial quality (Yang, Zhou, Yang, Wang, & Wang, 2008). During the last years, a renewed interest in enzymatic degumming has been observed (Jiang, Chang, Jin, & Wang, 2015a; Yang et al., 2008). The reason being mainly due to the fact that enzymatic degumming not only presents a very efficient and eco-friendly solution for the industrial process, but it is also a process that increases the refined oil yield (De Greyt, 2013).

Phospholipases are a class of hydrolytic enzymes that can hydrolyze the ester bonds of phospholipids. The main phospholipase types are A₁, A₂, C, and D, with their target sites varying according to their specificity. The most commonly used phospholipases are phospholipase A₁ (Jahani, Alizadeh, Pirozifard, & Qudsevali, 2008; Sampaio et al., 2015), and phospholipase A₂ (Clausen, 2001), which remove the fatty acid from position 1 and 2 with respect to glycerol. Recently, the use of phospholipase C (PLC) has been introduced in the industry (De Greyt &
K.A. Sampaio, et al.

2. Material and methods

2.1. Raw material and reagents

Crude corn oil was obtained from a European crushing plant. All chemicals used are either High Performance Liquid Chromatography (HPLC) or analytical grade. Sodium hydroxide (NaOH) and citric acid (CA) were purchased from Sigma Aldrich (Brussels, Belgium). Demineralized water was purchased from VWR (Brussels, Belgium). The diolein standard (purity ≥ 99%) was purchased from Sigma Aldrich (Brussels, Belgium).

2.2. Enzyme

The phospholipase C type Purifine® PLC was kindly donated by DSM Company (Delft, The Netherlands). The commercial sample originates from a selected strain of Pichia pastoris with an activity of 27.500 PLCU/g.

2.3. Physicochemical analysis

The free fatty acid content (FFA) was determined by titration according to the Official Method Ca 5a-40 (American Oil Chemists’ Society, 2012a), and was expressed as weight % oleic acid. Elements (P, Fe, Ca, and Mg) were measured by Inductively Coupled Plasma (ICP) according to Method Ca 20-99 (American Oil Chemists’ Society, 2012b). The pH of the gums was measured directly with a pH electrode into the gums fraction (Sartorius, Germany). The diacylglycerol (DAG) content was measured according to the Official Method Cd 11b-91 (American Oil Chemists’ Society, 2012c). The samples were analyzed through gas chromatography using an Agilent Technologies 7890A (USA) equipment. A DB-5HT capillary column (15 m × 0.32 mm i.d. X 0.10 μm film thickness) was used, and 1 μL of sample was injected. The analysis was performed under the following conditions: oven temperature 50 °C; 50–200 °C (15 °C/min); 200–290 °C (3 °C/min. held for 10 min); 290–360 °C (10 °C/min. held for 15 min); flame ionization detector (FID) 380 °C; carrier gas Helium (He). The diacylglycerols were identified using an authentic diolein standard, as described in the method.

2.4. Nuclear Magnetic Resonance (NMR) analysis

The phospholipid (PL) composition of the oils and gums was analyzed externally by a certified analytical laboratory (Spectral Service GmbH, Koln, Germany). The quantitative phospholipids analysis was performed by Nuclear Magnetic Resonance (NMR) employing a Bruker Avance III 600 MHz automatic spectrometer. Triphenyl phosphate was used as internal standard (Diehl, 2008).

2.5. Water degumming (WDG)

Crude corn oil (400 g) was heated until the oil reached the desired temperature (80 °C). After that, 3% w/w of distilled water was added to the oil, and the mixture was homogenized with mechanical stirring (350 rpm) during 15 min. The hydrated gums were separated by centrifugation (2000 × g for 15 min). The top layer and the gums were collected for analysis.

2.6. Caustic pretreatment (CP)

The purpose of caustic pretreatment is to find an optimal pH value for the maximal enzyme activity. Crude corn oil (400 g) was heated to 80 °C and sodium hydroxide (NaOH) was dosed as a 14% aqueous solution in amounts varying from 50 to 700 mg/kg, under high shear mixing (16,000 rpm) for 1 min. After homogenization, the mixture was allowed to mix for 15 min with stirring at 350 rpm. Subsequently, the oil was centrifuged (2000 × g for 15 min), and the supernatant/gums were analyzed.

2.7. Chemical conditioning (CC)

The chemical conditioning is an alternative to reach an optimal pH value for maximal enzyme activity. However, in this case a citrate buffer is formed in situ. Crude corn oil (400 g) was heated to 80 °C, and citric acid (CA) was dosed as a 30% aqueous solution in an amount of 900 mg/kg. The mixture was treated for 1 min at 16,000 rpm and was further agitated for 15 min at 350 rpm. After that, an aqueous solution of sodium hydroxide (14%) was added in amounts varying from 840 to 1,300 mg/kg, on dry basis, covering three different molar ratios CA/NaOH (1/1; 1/1.25; 1/1.50). High shear mixing (1 min/16,000 rpm) was applied to the oil in order to disperse the caustic solution. Afterwards, the gums were separated from the degummed oil by centrifugation (2000 × g for 15min), and both were sent for analysis. The same procedure was applied to optimize the chemical concentration (CA/NaOH) for the chosen molar ratio (1/1).

2.8. Enzymatic degumming experiments (EDG)

The enzymatic degumming experiments were performed by using 400 g of crude corn oil. The first step of the enzymatic degumming process using Purifine® PLC was carried out similarly to the caustic pretreatment (CP) or chemical conditioning (CC) step in order to adjust the pH. Therefore, after 15 min of oil conditioning at 80 °C with stirring at 350 rpm, the temperature of the oil mixture was decreased to 50–65 °C. Then, a certain amount of water (3%, relative to the weight of the oil), and a predefined quantity of PLC (0–400 mg/kg) were added. The mixture was homogenized under high shear (16,000 rpm) for 1 min to disperse the enzyme in the oil/water emulsion. The oil mixture was maintained at the required temperature under gentle mixing (350 rpm) for a given time (15–240 min). The degumming reaction was stopped by heating the sample for 15 min at 85 °C. Subsequently, the oil mixture was centrifuged (2000 × g for 15 min) to separate the degummed oil from the gums.

2.9. Statistical analysis

All measurements were performed in triplicate with all data expressed as mean value ± standard deviation of independent triplicate experiments. Statistical analysis was performed with Origin 8.0.
The addition of enzyme (Puri® solution in water. The experiments were performed with and without – added in amounts corresponding to 50 phosphorus content was reduced to 67 mg/kg and the gums fraction of DAG increase of 0.59%. During the water degumming process the total of 1.66% phospholipids. Hence resulting in a potential DAG ab-

phatidylethanolamine (PE) represents 0.86% of the oil that contains a fi

tion with Puri

crease of the caustic concentration. However, after 120 min of reac-

tion with Purifine® PLC, the pH of the gums showed a considerable decrease, especially in the caustic concentration ranging from 300 to 500 mg/kg, which corresponds to a pH range of 5.4–6.0. Higher caustic concentrations (600–700 mg/kg phosphorus).

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Crude Corn Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA (%)</td>
<td>2.30 ± 0.10</td>
</tr>
<tr>
<td>DAG (%)</td>
<td>1.99 ± 0.06</td>
</tr>
<tr>
<td>Fatty acid composition (%)</td>
<td></td>
</tr>
<tr>
<td>Palmitic (C 16:0)</td>
<td>11.72 ± 0.32</td>
</tr>
<tr>
<td>Palmitoleic (C 16:1)</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Margaric (C 17:0)</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Stearic (C 18:0)</td>
<td>1.74 ± 0.10</td>
</tr>
<tr>
<td>Oleic (C 18:1)</td>
<td>29.61 ± 0.35</td>
</tr>
<tr>
<td>Linoleic (C 18:2)</td>
<td>55.27 ± 0.58</td>
</tr>
<tr>
<td>Linolenic (C 18:3)</td>
<td>0.89 ± 0.15</td>
</tr>
<tr>
<td>Arachidic (C 20:0)</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>Gadoleic (C 20:1)</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

Elements (mg/kg)

PC 69 ± 0.04
PE 0.17 ± 0.02
PI 0.35 ± 0.01
PA 0.20 ± 0.03
Others 0.25 ± 0.02
Total 1.66 ± 0.05

Results of FFA, DAG, Fatty acid composition, Elements, and Phospholipids composition.
Content are the mean of triplicate ± S. D.

(OriginLab Ltd., USA). Differences among the means were determined using Tukey test. Significant differences were declared at P ≤ 0.05.

3. Results

The phospholipid composition, free fatty acid content, fatty acid composition, diacylglycerols, and trace elements content of crude corn oil are listed in Table 1. Corn germ oil has linoleic acid as the predominant fatty acid, followed by oleic, and palmitic acid, with minor quantities of other fatty acids. The total phosphorus content, determined by inductive coupled plasma (ICP) is at a level of 951 mg/kg, while the phospholipids content evaluated by 31P-NMR reaches 1.66% (664 mg/kg phosphorus).

Phosphatidylcholine (PC), which is known as the strongest emulsi-

fer (Miura, Mutoh, Shinoki, & Yoshioka, 2006), together with phos-
phtidylethanolamine (PE), the pH of gums showed a considerable
duction in the degummed corn oil (Fig. 1). Taking into account the total content of PC and PE, the maximal relative DAG increase obtained at the optimal pH (5.7) corresponds to 62%, considering the possible hydrolysis of the PC and PE present.

Compared to the control caustic pretreatment, and the traditional water degumming process (3% water, 350 rpm), a decrease of the residual phosphorus content was found when performing the experiment with Purifine® PLC, after caustic pretreatment (Table 2). However, by increasing the caustic concentration the residual phosphorus tends to increase.

3.1. Enzymatic degumming with caustic pretreatment

A first attempt to reach the optimal pH for the enzyme addition was carried out using a caustic pretreatment (CP). Sodium hydroxide was added in amounts corresponding to 50–700 mg per kg of oil as a 14% solution in water. The experiments were performed with and without the addition of enzyme (Purifine® PLC) in order to follow the changes of the pH of gums, DAG increase and residual phosphorus content within a reaction time of 120 min.

Fig. 1 shows the pH of corn oil gums as a function of the addition of different caustic concentrations. The pH of the gums increased with the increase of the caustic concentration. However, after 120 min of reaction with Purifine® PLC, the pH of the gums showed a considerable decrease, especially in the caustic concentration ranging from 300 to 500 mg/kg, which corresponds to a pH range of 5.4–6.0. Higher caustic concentrations (600–700 mg/kg) resulted in smaller pH decreases when comparing the gums with and without Purifine® PLC addition.

There is a clear correlation between the pH of the gums and the DAG increase in the degummed corn oil (Fig. 2). According to the data, optimal pH for Purifine® PLC applied to crude corn oil seems to be within the range of 5.4–6.0, with an optimal value at pH 5.7. Similar results were obtained for rapeseed oil (Ye et al., 2016), and camellia oil (Jiang et al., 2015b) when using a PLC enzyme originating from Bacillus cereus. Taking into account the total content of PC and PE, the maximal relative DAG increase obtained at the optimal pH (5.7) corresponds to 62%, considering the possible hydrolysis of the PC and PE present.

3.2. Enzymatic degumming with chemical conditioning

A chemical conditioning (CC) consisting of the addition of citric acid (CA) and NaOH was applied as a pretreatment. Since the CC was not established before, experiments were carried out with crude corn oil using three different molar ratios (1/1, 1/1.25, and 1/1.50) of citric acid/sodium hydroxide. The pH of gums obtained from a molar ratio of 1/1, 1/1.25, and 1/1.50 was 5.7, 6.0, and 6.5, respectively. Therefore, only the first molar ratio (1/1) was optimized in view of the amount of chemicals, which is intrinsically related to the cost of the process and environmental concerns.

Fig. 3a shows the effect of citric acid on the Purifine® PLC reaction at a molar ratio of CA/NaOH (1/1), at a temperature of 60 °C, and a re-

action time of 120 min. The residual phosphorus content of corn oil treated by Purifine® PLC showed a decrease with an increase in the

Fig. 1. Effect of caustic pretreatment (CP) and caustic pretreatment plus en-

zyme (CP- Purifine® PLC) addition on the pH of corn gums.

Fig. 2. Effect of the pH of corn oil gums on relative DAG increase.

Fig. 3a. Effect of citric acid on the Puri-

ne® PLC addition.
amount of CA (500–900 mg/kg), while the DAG content increased with the increase of CA. From these results, it can be concluded that 900 mg/kg of CA is the most convenient concentration for chemical conditioning and this was then used for further experiments.

With the optimal pH fixed at 5.7, the effects of reaction temperature, Purifine® PLC dosage, and reaction time on the residual phosphorus content and DAG increase were studied. Fig. 3b shows the effect of the reaction temperature on DAG increase and residual phosphorus content (P-content) of the degummed corn oil. By increasing the temperature from 50 to 60 °C, the content of residual phosphorus decreases, while the relative DAG content increases. However, at temperatures higher than 60 °C the DAG content was decreased. Based on the obtained results it was established that the most suitable temperature for the highest enzyme activity was 60 °C (Jiang et al., 2014), previously performed enzymatic degumming studies on soybean oil at different temperatures using a PLC enzyme from a genetically modified Bacillus cereus and reported that the lowest residual phosphorus (15.6 mg/kg) was obtained at 55 °C.

Fig. 3c shows the effect of Purifine® PLC dosage on DAG increase and residual phosphorus content of degummed corn oil. With an increase in Purifine® PLC dosage from 100 to 200 mg/kg, the relative DAG content increases linearly. The tendency to increase became very slow with further addition of enzyme (300–400 mg/kg). On the other hand, the residual phosphorus content first decreased with the addition of enzyme, and then slightly increased. Jiang et al. (2015b) obtained similar results when performing the optimization of the degumming process of camellia oil.

Fig. 3d shows the effect of reaction time on the relative DAG increase and residual phosphorus content of the degummed corn oil. With the addition of 200 mg/kg Purifine® PLC, the amount of DAG showed a relative increase of 92% within 120 min, while the residual phosphorus content could be reduced to 27 mg/kg. Further increase on the reaction time (240 min) contributed only to a slightly increase of the DAG content, and decrease of the residual phosphorus. Considering these results, 120 min reaction time was fixed as an optimum value for the enzymatic degumming of corn oil.

3.3. Evaluation of the phospholipid composition

The phospholipid composition of the three different degummed oil gums was analyzed by 31P-NMR, and the results are presented in Fig. 4. As can be seen, in water degummed oil gums, PC is the phospholipid with the highest concentration (39.1%), followed by PI (24.7%), while the content of PE and PA were the lowest (10.8% and 8.9%,

![Fig. 3a. Optimization of citric acid/sodium hydroxide concentration for a constant molar ratio (1/1), reaction time of 120 min and temperature of 60 °C.](image)

![Fig. 3b. Effect of temperature on the residual phosphorus content and DAG increase (reaction conditions: 120 min; 60 °C; 3% water; pH 5.7).](image)

![Fig. 3c. Effect of Purifine® PLC dosage on the residual phosphorus content and DAG increase (reaction conditions: 120 min; 60 °C; 3% water; pH 5.7).](image)

![Fig. 3d. Effect of reaction time on the residual phosphorus content and DAG increase (reaction conditions: 200 mg/kg Purifine® PLC; 60 °C; 3% water; pH 5.7).](image)
respectively. According to (Dayton & Galhardo, 2014), in water degumming, the Ca, Mg, and Fe salt forms of PA and PE are not hydratable, and therefore remain in the oil.

In Purifine® PLC degummed oil gums obtained after caustic pretreatment (CP-PLC), the content of PC and PE were 14.7% and 6.8%, respectively. However, the relative content of PI and PA (41.9% and 16.7%, respectively) increased as compared to water degumming. A similar result was observed when performing Purifine® PLC degumming (EDG-PLC) with the aid of chemical conditioning. In these experiments, the PC content was almost completely consumed (< 1.4%), and PE was reduced to 6.1%, while the content of PI and PA were 49.1% and 19.0%, respectively.

Fig. 5 presents the decomposition of the phospholipids in the gums fraction over time under the optimized degumming conditions (200 mg/kg Purifine® PLC, 60 °C, 120 min, and pH 5.7). After 30 min of reaction, about 60% of PC and PE were hydrolyzed, confirming the fast enzyme kinetics. The hydrolysis seems to occur until 90 min, and only small levels were detected at 120 min. It should be highlighted that, as most PC and PE were hydrolyzed, the relative content of PI and PA steadily increased with the reaction time.

4. Discussion

The production of refined corn oil requires the removal of phospholipid from the crude vegetable oil. In this work, the total phosphorus content (951 mg/kg) which takes into account inorganic phosphorus, glycerophosphates, and phospholipids presented a substantial discrepancy when comparing to the phosphorus (664 mg/kg) coming only from the phospholipids. For instance, the knowledge of the phospholipid content and composition is crucial for evaluating the effectiveness of the degumming processes, the expected oil yield increase, and finally the feedstock quality (De Greyt & Kellens, 2005).

During the water degumming process, most of the hydratable phospholipids (HPL) are removed with water by centrifugation, and the residual phosphorus content of corn oil was 67 mg/kg. According to Lamas, Constenla, & Raab (2016), the phospholipids remaining after the water treatment can be considered as non-hydratable phospholipids (NHPL). It has also been reported that phosphatidic acid (PA) has a great affinity for divalent ions, such as Ca and Mg, and their salts formed are the NHPL present in the oil (Dijkstra, 2011).

According to the supplier data, Purifine® PLC has an optimum pH ranging from 5.5 to 5.9. However, the pH of the water degummed gums has a value of 4.4, indicating a clear need of pH adjustment before enzyme addition. The pH adjustment can be performed using caustic soda or through the use of a buffer formed in situ. However, according to Tatulian (1993), the phosphate groups liberated by PC and PE have a pKₐ ≤ 3.5, thus possibly reacting with the sodium hydroxide solution, therefore lowering the pH of the gums. The mentioned issue could be avoided by using a citrate buffer, which stabilizes the medium.

The relative DAG content (Fig. 2) seems to have a direct correlation with the pH, while the residual phosphorus content (Table 2) is more related to the phospholipids dissociation and hydratability. In fact, Purifine® PLC hydrolyzes the bond between the acylglycerol and the phosphate groups present in PC and PE, liberating diacylglycerols (DAG), which will not be removed from the oil, then contributing to the oil yield increase (Dayton & Galhardo, 2008). However, by using a caustic pretreatment, an incomplete DAG conversion was obtained, which can be attributed to the pH change during the degumming reaction. As stated by Yang et al. (2008), each kind of enzyme has its optimal pH range, and values outside this range can lead to a partial or complete inactivation of the enzyme.

Crude oil, either water degummed or not, is treated with an acid, usually phosphoric or citric acid, in the presence of water. The citric acid forms a complex with calcium and magnesium decomposing the NHPL, and the subsequent addition of caustic soda causes the released NHPL to dissociate and become hydratable (Dijkstra, 2011). In
addition, higher concentrations of chemicals (Fig. 3a) will influence the effectiveness of a buffer, therefore avoiding pH changes and favoring a better enzyme performance in terms of the degumming yield. As reported by (More & Gogate, 2018), the effect of pH is essentially related to the enhancement in ionization degree and availability of more active sites on enzyme surface.

There are several factors that affect the kinetics of enzyme activity, such as pH, temperature, enzyme loading, and reaction time. Enzyme activity occurs within a narrow range of temperatures compared to chemical reactions. The rise of the temperature within the optimal range can improve the reaction rate due to a reduced viscosity of the lipid mixture and a better enzyme-substrate contact. In contrast, temperatures higher than the enzyme optimal value can cause partial denaturation of the enzyme with loss of its hydrolytic activity (Jiang et al., 2014).

Regarding the enzyme loading, it is known that if the concentration of the enzyme is increased, the velocity of the reaction proportionally increases (Robinson, 2015). However, when the substrate is already consumed, the excess of enzyme will only increase costs. In fact, the enzyme Purifine® PLC acts specifically towards PC and PE, and an enzyme dosage of 200 mg/kg is sufficient to bring down these phospholipids to a minimum value within a reaction time of 120 min, indicating that the enzyme works fast.

The evaluation of the phospholipid composition of the different gums fractions is essential to study the conversion of some kinds of phospholipids into diacylglycerols and phosphate groups under appropriate conditions. As the enzyme Purifine® PLC is highly selective towards PC and PE, there will be a relative increase of PI and PA in the gums fractions. Therefore, this process can be a good alternative to traditional degumming processes.

The authors declare no conflict of interest.

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