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IMMOBILIZATION OF PHOSPHOLIPASE A1 USING A POLYVINYL ALCOHOL-ALGINATE MATRIX AND EVALUATION OF THE EFFECTS OF IMMOBILIZATION

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Abstract - The paper presents the synthesis and performance of an immobilized phospholipase A1 with practical application for oil degumming. The polyvinyl alcohol (PVA) had a number of properties indicating this polymer as a good enzyme carrier. The combination with alginate made a macro-porous structure, evidenced by SEM analyses. When the process time in boric acid solution was 30 minutes, the results revealed that beads prepared with 10% (w/v) PVA and 2% (w/v) sodium alginate in 4% (w/v) boric acid and 2% (w/v) calcium chloride solution exhibited high immobilized enzyme activity, immobilization yield and stability. The pH and temperature optimum for the PVA–alginate immobilized phospholipase A1were 5.6 and 58 °C, respectively. The enzyme immobilized in the beads retained 50.37% of the initial activity in the eighth cycle. The enzyme biocatalyst immobilized in the beads retained 78.58% of the initial activity after storing 6 weeks at 4 °C.
Keywords: Phospholipase A1; PVA; Sodium alginate; Immobilization; Beads stability.

INTRODUCTION

The method of enzymatic degumming of oil was first developed in the 1990's in initial industrial plant trials by the German Lurgi Company as the "EnzyMax process" (Aalrust *et al*., 1992), which has been widely applied all over the world. The reaction is a hydrolysis of the phospholipids, turning them into fatty acids and the water-soluble form. The water-soluble form makes them easy to remove with the water phase. Enzymatic degumming of vegetable oil was much more effective and environmentally friendly than traditional methods. The amounts of acid and base used, wastewater discharged and cost in the process of enzymatic degumming were reduced distinctly and the product yield highly increased (Dahlke, 1998). Besides oil degumming, the enzyme has been used in other fields such as enantioselective reactions (Mishra *et al*., 2009) and the improvement of lecithin emulsification properties (Yang *et al*., 2008).

Much attention has been paid to enzyme immobilization and has focused on the advantages of the immobilized enzyme compared with free enzyme, including protection from harsh environmental conditions, reusability and relative ease of product separation etc. Different matrices have been studied for the efficacy of phospholipase immobilization, such as vinyl-polymer (Bajpai and Bhanu, 2003),

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alginate-silicate sol-gel matrix (Kim *et al*., 2001), gelatin hydrogel (Freitas *et al*., 2012; Sheelu *et al*., 2008), chitosan beads (Chen and Chen, 1998; Adriano *et al*., 2005) or agarose beads (Ferreira *et al*., 1993; Shen and Cho, 1995). Each matrix has its own advantages and disadvantages for the enzyme immobilization. A suitable matrix for enzyme immobilization is required to retain high immobilized enzyme activity, high immobilization yield, high stability and low cost. Polymeric materials are often selected to immobilize enzymes for biological treatment. Compared with other immobilization materials, polyvinyl alcohol (PVA)–alginate based matrices boast many good properties for parameters such as the immobilized enzyme activity, reusability and stability. It was presumed that PVA might contribute to improve the durability and strength of the beads, while sodium alginate might improve the surface properties of the beads, reducing the tendency to agglomeration (Long *et al*., 2004). PVA–alginate beads have been used as a carrier for immobilized cells and enzymes (Idris *et al*., 2008; Nunes *et al*., 2010; Qin *et al*., 2010; Takei *et al*., 2011). However, very little literature on PVA–alginate beads as an immobilization material of phospholipase A1 is available. Immobilized phospholipase A1 has been used in oil substrate, but PVA–alginate beads have been used primarily in aqueous media.

The aim of this paper was to investigate the feasibility of employing PVA–alginate beads treated with boric acid solution for oil degumming. In addition, the effects of PVA–alginate on phospholipase A1 immobilization were evaluated by testing parameters such as immobilized phospholipase activity, immobilization yield and stability. The immobilization effects of PVA–alginate beads under different preparation conditions such as matrix concentration, crosslinkage solution composition, enzyme load, temperature, pH and stability were compared, and the optimum conditions were selected.

MATERIALS AND METHODS

Materials

Lecitase Ultra (EC 3.1.1.3) from Thermomyces lanuginosus/Fusarium oxysporum was purchased from Novozymes A/S; the protein content was 4 mg/g and activity was 10000U/g. Polyvinyl alcohol (1750±50) and other chemical reagents were purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). Crude rapeseed oil was physical squeezed in the laboratory.

Determination of Phospholipase Activity

Free phospholipase activity was determined with a phospholipid emulsion, generally according to Yang *et al*. (2006). One unit of phospholipase (U) is the amount of enzyme which releases 1 umol of titratable free fatty acids (FFA) per minute.

Substrate solution: 5% phospholipid and 2.5% polyvinyl alcohol solution was mixed at a ratio of 4:1 (v/v) at 10 000 rpm for 10 min to emulsify. The solution pH was adjusted with citric acid and disodium hydrogen phosphate. Enzyme solution (10 μL) was added to the substrate solution (30 mL) and incubated at 50 °C for 10 min. The reaction was terminated by the addition of 95% ethanol (15 mL) after incubation, and the liberated fatty acids were titrated with 0.1 mol/L NaOH. Blanks were measured with an enzyme stock solution which was kept at 100 °C for 15 min and the solution was used for the active enzyme sample after cooling to ambient temperature.

Immobilized phospholipase A1 activity in polyvinyl alcohol (PVA)-alginate beads was measured by the same method as above for free enzyme, but using the Immobilized phospholipase A1 (0.2 g) as a catalyst.

Preparation of Immobilization Beads

The solution of PVA (ranging from 8% to 12%) (w/v) and sodium alginate (ranging from 0.5% to 4.0%) (w/v) was heated to 80 °C to completely dissolve the PVA and sodium alginate and then cooled to room temperature. A certain volume of phospholipase A1 solution was added and mixed in the solution thoroughly. The solution was extruded from a needle (diameter 0.8 mm) into a mixed solution of 1000 mL boric acid (2% to 6%, w/v) and calcium chloride (0.1 to 4%, w/v), and immersed for 10-50 min, forming beads.

The beads were washed with distilled water to remove any excess of boric acid and kept at 4 °C to the complete solidification for further use.

Determination of PVA Bead Stability

Mechanical strength was determined as per the method of Chang *et al*. (1998) with little change. The relative mechanical strength of the PVA-alginate beads was tested using a 2-blade turbine and a cylindrical beaker with four baffles (1 cm wide each). Fifty beads were added to the beaker with oil (50 °C) to 5 cm in height. The agitation speed was controlled from 500 to 3000 rpm. The beads were agitated in the beaker for 5 minutes, and the surviving beads were counted. Each run was performed three times.

Immobilization Efficiency

Immobilization yield is defined as the ratio of the activity of immobilized enzyme to the activity of the free enzyme used (Idris *et al*., 2008).

$$\text{Immobilization yield (\%)} = \frac{\text{activity of immobilized enzyme}}{\text{activity of free enzyme}} \times 100$$

$$\text{Immobilization enzyme activity (U / g)} = \frac{\text{enzyme activity of the beads}}{\text{quantity of beads}}$$

Optimum pH and Temperature

The optimum pH of both free and immobilized phospholipase A1 was studied using phospholipid emulsion with pH values ranging from 3.6 to 6.8, at 50 °C, 150 rpm, and incubation for 10 min.

The optimum temperature of both free and immobilized phospholipase A1 was determined by incubation at various temperatures (from 35 to 65 °C), pH 5.0, at 150 rpm, for 10 min.

In both cases, the enzyme activity was measured.

Operational and Storage Stability

In the recycle studies, the immobilized phospholipase A1 was used eight times. Each conical flask was shaken on an orbital shaker with crude rapeseed oil at 150 rpm and 50 °C for 3 h. The crude rapeseed oil was decanted and replaced with fresh oil. The studies on 8 recycles were determined with the same batch of immobilized enzyme.

The stability of immobilized phospholipase A1 beads was monitored during 6 weeks at 4 °C. The residual activity was measured.

Observation by Scanning Electron Microscopy (SEM)

The PVA–alginate beads were dried and cut with a knife to get the cross-section of the bead. Then the surface and the cross-section of the beads were sputter-coated with gold and the surfaces and cross-section were obtained using SEM (JSM-6490LV).

Statistical Procedure

All experiments were repeated at least 3 times, and the results were expressed as means ± standard deviations. Data were analyzed using SPSS 17.0.

RESULTS AND DISCUSSION

Effect of Different Times of Immersion in Cross-Linkers on Efficiency of Immobilization

The equilibrium between mechanical stability of the beads and immobilized enzyme activity has to be considered. In order to form beads with durability and strength, the aqueous solution containing PVA $(–CH_2CHOH–)_n$ was added to the boric acid solution containing $B(OH)^{4–}$ ions. These borate ions cross-link with the alcohol groups on adjacent chains (Idris *et al*., 2008). Calcium alginate was formed as beads almost instantaneously when the sodium alginate came into contact with calcium chloride solution (Grishin and Tuovinen, 1989). However, saturated boric acid solution was highly acidic (pH < 4) and caused a drastic decrease in the activity of immobilized enzyme.

Table 1 shows the high immobilized enzyme activity and immobilization yield of phospholipase A1 in the immobilized forms with 30 minutes of exposure time to boric acid. The immobilized enzyme activity and immobilization yield decreased with decreasing exposure time up to 30 minutes due to the low degree of physical crosslinking of PVA. After 30 minutes of exposure to boric acid, however, the immobilized enzyme activity and immobilization yield decreased with increasing exposure time because of the high acidity of boric acid solution.

Table 1: Immobilized enzyme activity and immobilization yield with different times of exposure to 4% boric acid extrusion solution during immobilization.

Time (min)	Immobilized enzyme activity (U/g)	Immobilization yield (%)
10	NF	NF
20	85.48±1.77c	68.80±1.42b
30	94.78±1.46a	74.12±1.14a
40	91.00±1.62b	69.76±1.24b
50	88.78±1.62b	62.93±1.15c

NF: the bead could not be formed in this condition

The beads treated for 20-50 minutes had a very strong mechanical strength and are very difficult to break. They were not broken even at agitation speeds of 3000 rpm for 5 minutes (data not shown). For the equilibrium between mechanical stability and immobilized enzyme activity of the beads, the

optimum process time in boric acid solution was 30 minutes.

Candida rugosa lipase (Lipase AY-30) was immobilized by covalent binding on poly(γ-glutamic acid) (γ-PGA). The optimum immobilization time was 2.3 h (Chang *et al*., 2007).

The Effects of Different Preparation Conditions of PVA–Alginate Beads on the Immobilization

The preparation conditions such as concentration of matrix (PVA and sodium alginate) and cross-linkage (boric acid and calcium chloride) solution, need to be properly established in order to improve the efficiency of enzyme immobilization, since different preparation conditions could highly affect the immobilized enzyme activity and immobilization yield (Idris *et al*., 2008; Nunes *et al*., 2010).

Enzyme immobilized in the beads treated with high concentrations of boric acid showed low activity and immobilization yield. Low concentrations of boric acid could increase the immobilized enzyme activity and immobilization yield (Table 2). The result could be accounted for by the excess borate ions, which could create an acidic micro-environment inside the beads and decrease the enzyme activity and immobilization yield. Meanwhile, higher boric acid concentrations resulted in denser beads by forming smaller pores in the beads, which could limit the diffusivity of the substrate and in turn reduce the immobilized enzyme activity and immobilization yield (Idris *et al*., 2008). For the equilibrium between immobilized enzyme activity and immobilization yield, the concentration of 4% was selected as the best boric acid concentration for the bead preparation.

Table 2 also shows the high immobilized enzyme activities and immobilization yield of phospholipase A1with 2% calcium chloride solution. The immobilized enzyme activity and immobilization yield decreased with decreasing concentration of calcium chloride below 2%, which may be caused by the decrease in the mechanical strength of the sodium alginate gel with the decrease of Ca^{2+} concentration (Chen *et al*., 1996). When the concentration of calcium chloride exceeded 2%, however, the immobilized enzyme activity and immobilization yield decreased with increasing calcium chloride concentration, which implied that excessive gelation of sodium alginate formed on the surface limited the diffusivity of the substrate and in turn reduced the immobilized enzyme activity and immobilization yield.

The highest immobilized enzyme activities and immobilization yield of phospholipase A1 were observed at the concentration of 10% PVA (Table 3). Increasing of the concentration of PVA from 8% to 10%, the immobilized enzyme activity and immobilization yield increased. The results suggest that the amount and size of the macropores decreased and the thickness of the pore walls increased (Lozinsky and Plieva, 1998). The immobilized enzyme activity and immobilization yield decreased when the concentration of PVA continued to increase to 12%. The results could be explained by the smaller pores in the beads limiting the diffusivity of the substrate.

The highest immobilized enzyme activities and immobilization yield of phospholipase A1 were found at the sodium alginate concentration of 2% (Table 3). The immobilized enzyme activity and immobilization yield decreased with decreasing concentration of sodium alginate below 2%. The result could be explained by a low crosslinking rate of PVA. Increasing the concentration of sodium alginate from 2% to 4%, the immobilized enzyme activity and immobilization yield decreased. The result suggest that the number of hydrogen bonds between polymer chains increased, which led to an increase in the density of physical cross-linking sites (Nunes *et al*., 2010; El-Din *et al*., 2007).

Table 2: Immobilized enzyme activity and immobilization yield with different concentration of boric acid and calcium chloride solution during immobilization.

Boric acid (%)	Immobilized enzyme activity(U/g)	Immobilization yield(%)	Calcium chloride (%)	Immobilized enzyme activity(U/g)	Immobilization yield(%)
2	NF	NF	0.1	82.17±2.12d	69.74±1.80c
3	98.84±2.79a	70.36±1.35b	0.5	85.16±2.14cd	72.12±1.81c
4	95.21±2.57a	75.25±2.03a	1.0	92.89±1.83b	76.36±1.50b
5	76.47±2.67b	70.02±2.44b	2.0	101.64±1.66a	80.42±1.31a
6	64.46±2.43c	62.37±2.35c	4.0	87.45±1.99c	77.87±1.78ab

NF: the bead could not be formed in this condition

Table 3: Immobilized enzyme activity and immobilization yield with different concentration of PVA and sodium alginate during immobilization.

PVA (%)	Immobilized enzyme activity(U/g)	Immobilization yield(%)	Sodium alginate (%)	Immobilized enzyme activity(U/g)	Immobilization yield(%)
8	80.15±1.82d	65.20±1.48c	0.5	48.17±1.19e	35.99±0.89e
9	90.48±2.18b	71.35±1.72b	1.0	105.24±1.75b	80.27±1.33b
10	103.51±1.85a	81.23±1.45a	2.0	126.77±3.04a	84.20±2.02a
11	91.68±2.51b	78.70±2.15a	3.0	75.04±2.42c	57.09±1.84c
12	85.67±2.27c	72.00±1.91b	4.0	62.13±2.22d	51.93±1.85d

Effect of Different Concentrations of Enzyme on Activity

Table 4 indicated the effects of different concentrations of phospholipase A1 immobilized in PVA-alginate beads. The immobilized enzyme activity of the beads was positively linearly related to the enzyme concentrations from 1 to 5 g of enzyme per 100 g PVA–alginate hydrogel. However, the immobilization yield of the beads was negatively linearly related to the concentration of enzyme. The equilibrium point between immobilized enzyme activity and immobilization yield was found at the concentration 1 g of enzyme per 100 g PVA–alginate hydrogel, at which the effect of the phospholipase A1 immobilization is the best. A similar immobilization yield was reported for free phospholipase A1 in gelatin hydrogel (> 80%) (Sheelu *et al*., 2008).

Table 4: Immobilized enzyme activity and immobilization yield with different concentration of enzyme during immobilization.

Concentration of enzyme (g/100 g)	Immobilized enzyme activity (U/g)	Immobilization yield (%)
1	126.50±2.89d	85.31±1.95a
2	147.63±3.11c	63.41±1.33b
3	151.47±2.81c	46.60±0.86c
4	203.14±7.02b	43.58±1.51c
5	217.58±8.55a	41.78±1.64d

Effect of pH and Temperature on Enzyme Activity

The effects of pH on the activity of free and immobilized phospholipase A1 are shown in Fig. 1. It was observed that the optimum pH for free and immobilized phospholipase A1 were 5.2 and 5.6, respectively. It was also observed that high activity of the immobilized enzyme occurred in a broad pH between 5.2-6.4, which implied that the beads played a protective role for the immobilized enzyme. Similar results were reported for free phospholipase A1 in gelatin hydrogel (Sheelu *et al*., 2008), free naringinase in PVA matrices (Şekeroğlu *et al*., 2006; Nunes *et al*., 2010), laccase in PVA Cryogel Type Carrier (Stanescu *et al*., 2010) and dextranase in BSA with a cross-linking agent (El-Tanash *et al*., 2011).

It can be observed from Fig. 2 that the optimum temperature for the immobilized enzyme (58 °C) was approximately 8 °C higher than that for free enzyme (50 °C). At the higher temperature, the PVA beads played a protective role for the enzymes immobilized in the beads. High stabilization by the restriction of the movement of the enzyme immobilized in the beads could cause the increase of the optimum temperature for the immobilized enzyme. Similar results were found for phospholipase A1 in gelatin hydrogel (Sheelu *et al*., 2008) and other enzymes immobilized in matrices (Altun and Cetinus, 2007; Busto *et al*., 2007).

Operational and Storage Stability

Fig. 3 shows the activity of the immobilized enzyme after eight cycles of operation. The immobilized phospholipase A1 retained 71.89% of its initial activity after five cycles of operation. In fact, the immobilized enzyme lost almost half of its initial activity after eight cycles of operation. Data on operational stability from previously reported studies involving PVA immobilization of naringinase showed that only 36% of the initial activity was retained after six reuses (Busto *et al*., 2007).

Fig. 4 shows the storage stability for different times at 4 °C.The residual activities of the immobilized phospholipase A1 after storing at 4 °C for 1, 2, 3, 4, 5, and 6 weeks were 100%, 98.18%, 91.14%, 86.35%, 83.86% and 78.58%, respectively. The results indicated that the immobilized enzyme still kept high activity after 5 weeks, and retained 78.58% of the initial activity even after 6 weeks.

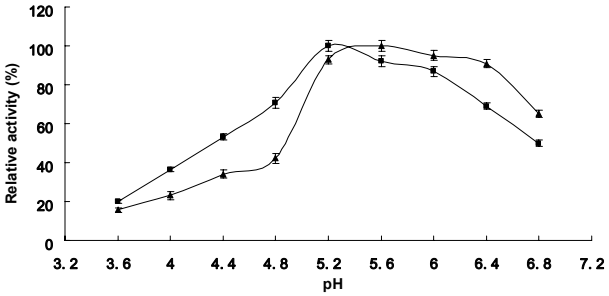

Figure 1: Relative activity versus pH, for free enzyme (■) and immobilized enzyme (▲) in PVA beads.

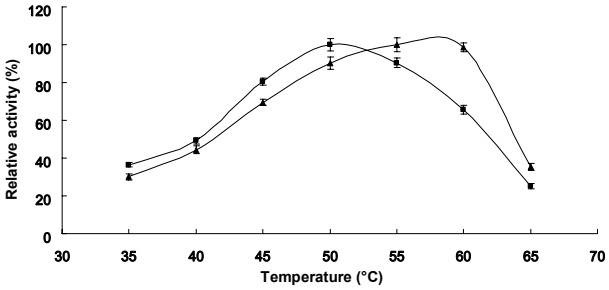

Figure 2: Relative activity versus temperature, for free enzyme (■) and immobilized enzyme (▲) in PVA beads.

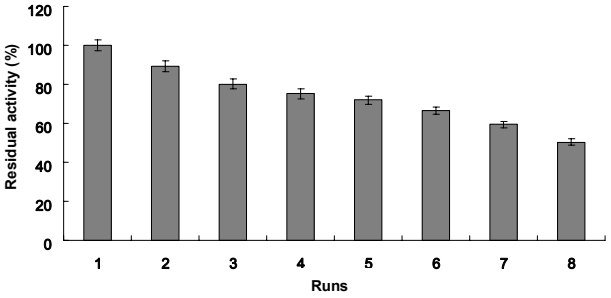

Figure 3: Recycling of immobilized phospholipase A1 in PVA– alginate beads.

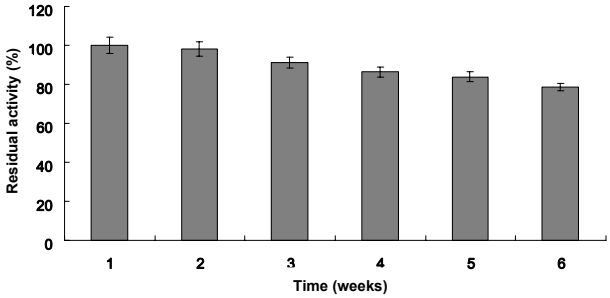

Figure 4: Storage stability of immobilized PVA–alginate beads.

Scanning Electron Microscopic Analysis

The PVA–alginate beads were observed and studied by SEM (Fig. 5). The cross-sectional image of the bead (Fig. 5a) indicated that clear layers of crosslinked polymers were formed inside the bead. The bead has a large internal cavity near the surface and a compact structure in the centre. A lot of little pores were formed on the surfaces of the beads (Fig. 5b). Fewer layers were formed near the surface of the beads (Fig. 5c) and a stable microstructure with evenly distributed layers was formed in the centre of the beads (Fig. 5d).

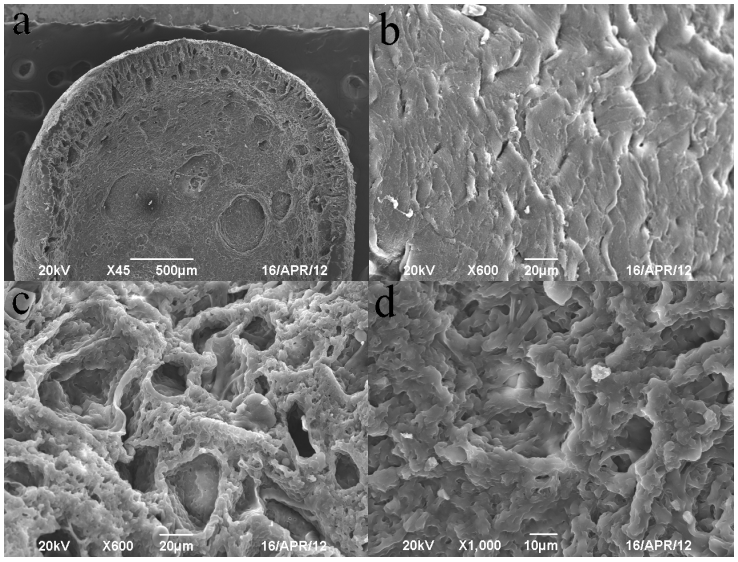

Figure 5: (a) Cross-section of PVA bead (b) magnified image of outer surface (c) near the outer surface (d) at the center of the bead.

The surface of the bead, with pores on it, appeared to be coarse and uneven, which is helpful to increase the specific surface area of the beads and increase the diffusion of substrates. Cross-sectional images of the beads indicated that the enzyme existed mainly in the submarginal sections of the beads with fewer enzyme in the center of the beads, which would make the enzyme in submarginal sections of the beads preferentially contact substrates and increase the activity of the immobilized enzymes.

CONCLUSIONS

The highest immobilized enzyme activity and immobilization yield were 126.50 U/g and 85.31%, obtained at the optimum conditions of 10% (w/v) PVA and 2% (w/v) sodium alginate matrix prepared with 4% (w/v) boric acid and 2% (w/v) calcium chloride solution for 30 minutes. 1 g enzyme per 100 g PVA–alginate matrix is the equilibrium point between immobilized enzyme activity and immobilization yield.

The optimum pH and temperature for the PVA–alginate immobilized phospholipase A1 were 5.6 and 58 °C, respectively. The enzyme immobilized in the beads was reused eight cycles and still retained 50.37% of the initial activity even in the last cycle. The activity of the enzyme immobilized in the beads retained 78.58% of the initial activity even after storing 6 weeks at 4 °C.

The polyvinyl alcohol (PVA)-alginate beads showed an excellent enzyme activity and mechanical stability in oil substrate at higher temperature. The images observed by SEM indicated that the structure of the beads was beneficial for increasing the activity of the immobilized enzymes. It could be concluded that the immobilization of phospholipase A1 using a polyvinyl alcohol-alginate matrix for oil degumming proved to be an effective technique and method.

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