IMMOBILIZATION OF DEGRADATIVE ENZYME ONTO COLLAGEN HYDROLYSATE FILMS*

by

C. SHANTHI, AND DENNIS C. SHELLY**

Leather Research Institute and Department of Chemistry and Biochemistry

TEXAS TECH UNIVERSITY, LUBBOCK, TX 79409

and

B. STENNETT

Tahoka Independent School District

TAHOKA, TX

ABSTRACT

Chemical or biological degradation of chrome shavings results in collagen hydrolysate and chrome cake. To add value to the recovered collagen hydrolysate, we evaluated films, cast from this material, for immobilizing degradative enzymes. Organophosphorus hydrolase (OPH) has been shown to be effective in degrading a range of organophosphate esters. Organophosphate compounds, which are among the most toxic substances known, are used as pesticides and nerve gases. A crude extract of OPH from *Flavobacterium sp.* was immobilized onto collagen hydrolysate films. The collagen films were characterized by differential scanning calorimetry and water swelling behavior. The properties of the collagen hydrolysate films were compared with the films cast from commercially available gelatin. The effect of glutaraldehyde crosslinking the films on enzyme immobilization was studied.

INTRODUCTION

The leather industry generates large quantities of solid wastes. The chrome-tanned wastes (shavings and trimmings) are suspect materials due to the perceived risk to the environment from the high chromium content. Some of the tanned wastes are used in the manufacture of leather boards[1] and the bulk is most often disposed in landfills. Landfill disposal of such wastes is not an overwhelmingly approved solution, as there exists a theoretical potential of chromium leaching, possibly contributing to ground water pollution. Hence, there is a need to develop useful products from these wastes for different commercial end uses to reduce the burden on landfills.

Removal or separation of chromium from these wastes has been advocated in order to utilize the protein for many other applications. Chrome-containing leather wastes can be degraded by chemical and enzymatic methods.[2,3] Efforts have been expended to employ the protein hydrolysate (resulting from degradation) as animal feeds, leather fillers and in organic farming. In the present investigation, we have attempted to use the protein hydrolysate as a support matrix for immobilization of a degradative enzyme, organophosphorus hydrolase (OPH). Collagen and its degraded product gelatin are considered ideal support matrices for the immobilization of enzymes. Enzyme immobilization is facilitated by the presence of myriad hydrophilic functional sites in the collagen matrix, on one hand, and its proteinaceous nature on the other. High water absorptivity of the matrix allows for sustaining the enzymatic activity as well.[4]

Soil microbes such as *Pseudomonas diminuta* and *Flavobacterium sp.* constitutively express the enzyme, OPH.[5] OPH is a metalloenzyme that has broad substrate specificity.[6] It degrades organophosphates ranging from pesticides to chemical warfare. It has potential applications in both demilitarization and decontamination.[7] Widespread enzyme use has been limited by the prohibitive cost of isolation and purification. In addition, it is very laborious to recover pure active enzyme from the reaction mixture. So, immobilization of enzyme onto supports i.e. for very large-scale bioreactors would be a valuable technique for increasing the life span of the biocatalyst. Reports of the use of materials such as nylon, porous glass and silica beads as supports for OPH immobilization have been published.[8,9] The goal is the development of enzyme reactors for detoxification of organophosphorus pesticides.

This paper describes the systematic investigation of collagen hydrolysate, obtained from the chemical hydrolysis of chrome shavings, as a support matrix for OPH immobilization.

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**Corresponding author

MATERIALS AND METHODS

Materials

Flavobacterium sp. strain 27551 was obtained from the American Type Culture Collection (Manassas,VA). Streptomycin sulfate, glutaraldehyde, gelatin (alkali extracted, Bloom value 225) and paraoxon were purchased from Sigma Chemical Co., (St. Louis, MO). All other reagents used were of analytical grade.

Methods

Partial Purification of OPH from *Flavobacterium sp.*

Organophosphorus hydrolase was partially purified from *Flavobacterium sp.* ATCC 27551. The cultures were grown in nutrient broth supplemented with 5g of glucose and 0.5 g of calcium nitrate per liter of broth. Liquid cultures were incubated at 30°C in a rotary shaker at 110 rpm for 48 hrs. The cells were harvested after 48 hrs, centrifuged at 5000xG for 20 min at 4°C. The cell pellets were resuspended in 15mL of 10mM potassium phosphate buffer of pH 7.2. The cell suspension was passed twice through a chilled French pressure cell (15,000 lb/in^2) and centrifuged at 10,000xG for 20 min at 4°C. The supernatant was subjected to 1% streptomycin sulfate precipitation to remove nucleic acids. Then the supernatant solution was precipitated with 45% ammonium sulfate. The precipitated protein was suspended in 10 mM potassium phosphate buffer and dialyzed against the same buffer. The dialysate was lyophilized and used as a crude enzyme for immobilization.

Preparation of Collagen Hydrolysate Films

The leather shavings were hydrolyzed according to an earlier procedure.[3] 300 mg of lyophilized collagen hydrolysate was solubilized in 10mL of distilled water in a magnetic stirrer. The solubilized solution was degassed and cast onto 15 x 10 cm plastic plates. The films were air dried for 48 hrs. Upon drying, the films were carefully removed from the plastic plate. The thickness of the films was measured with a Mitutoyo digital caliper. For comparison purposes gelatin was used as control. Gelatin films were cast from 3, 6 and 12 % aqueous solutions.

All films were characterized for their swelling properties and stability. The swelling behavior was studied by placing the films in an appropriate buffer, phosphate buffer pH 7.4 or tris buffer pH 8.5, for 24 hrs and the percent swelling was calculated as follows:

$$\frac{\text{Weight gain of films swollen in buffer for 24 hrs} \times 100}{\text{Initial weight of films}}$$

The stability of the films was determined by measuring thermal transitions using a differential scanning calorimeter. The hydrated specimens were blot dried with filter paper and immediately sealed in aluminum pans. The thermogram was recorded using a Shimadzu DSC-50 differential scanning calorimeter at the rate of 5°C per min from 30 to 300°C.

Effect of Crosslinking on Immobilization of Enzymes onto Films

To study the effects of glutaraldehyde crosslinking on immobilization, the films were cast in the wells of the microtiter plates. Crude enzyme was immobilized and crosslinked with either 1 or 2% glutaraldehyde for 10min. An enzyme assay was performed, in situ, for a minimum of 5 cycles with an interval of 24 hrs between each cycle. The enzyme was immobilized before and after crosslinking to determine the influence of crosslinking on the activity of the enzyme.

OPH Immobilization onto Collagen Hydrolysate and Gelatin Films

The films were swollen in 1mg/mL solution of crude enzyme preparation in either pH 7.2 phosphate buffer or pH 8.5 Tris buffer for 24 hrs at 10°C. The swollen films were then crosslinked in 1% glutaraldehyde for 10 min. The crosslinked films were rinsed several times in distilled water and stored in buffer solution at 10°C until use.

OPH Activity Assay

Paraoxon was the substrate for all enzyme activity assays. Enzyme activity was determined photometrically at 410 nm using a Dynatech Minireader II (Chantilly, VA) based on the absorption of 4-nitrophenol, the hydrolysis product of paraoxon. 195 µl of pH 8.5 Tris buffer and 5 µL of suitably-diluted enzyme preparation were added to the wells of the microtiterplates and incubated for 10 min. 20 mL of 0.5 mM paraoxon in 10% aqueous methanol was added to each well and incubated at 30°C for 10 min. The absorbance was then read at 410nm. Enzyme activity was expressed as international units (IU), where the IU is defined as the amount of enzyme required to liberate 1µmol of 4-nitrophenol per minute.

Immobilized OPH Activity Assay

The activity of immobilized OPH was assayed in similar fashion to that of the soluble enzyme, except a weighed quantity of enzyme-immobilized film was added instead of the soluble enzyme. The absorbance was read after removal of the immobilized enzyme from the solution. Activity yield was calculated as the ratio of the determined activity

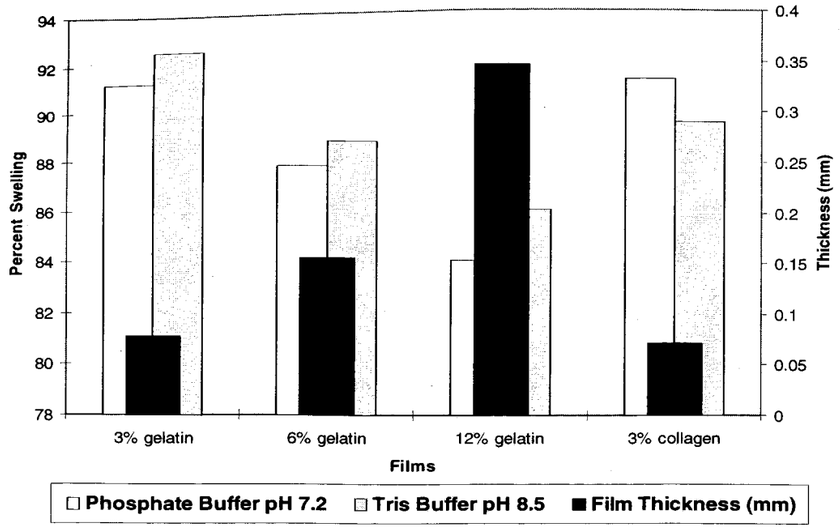


Figure 1. - Thickness and Swelling Percent of Prepared Films

of an aliquot of insolubilized enzyme to the same aliquot of initial active solution.

Results and Discussion

Collagen hydrolysate, obtained from chrome shavings, was assessed as a support matrix for immobilization of OPH enzyme. Comparisons were made to gelatin. Collagen hydrolysate after chromium removal was used without further purification. A 3%(w/v) solution of collagen hydrolysate was found to be optimum for casting films. Above this concentration, we experienced difficulty with solubility and below that the cast films were too thin to be removed from the plate without tearing.

Swelling Properties of the Collagen Hydrolysate Film

Film thickness was measured and the swelling behavior of the films was studied at pH 7.2 (open bars) and 8.5. (shaded bars) Results are presented in Figure 1. The thickness of the cast films with equal concentration of collagen hydrolysate and gelatin was almost equal as is evident from Figure 1. Similarly, the swelling behavior of the films for both the systems was identical at pH 7.2, whereas, at pH 8.5 the gelatin films (3% solution) swelled more extensively compared to collagen hydrolysate films. Since OPH is stable at neutral pH, the swelling behavior of the films was studied close to this pH (7.2 and 8.5) in appropriate buffer solutions. It has been reported that collagen film swelling can reach 500% in highly acidic and basic pH conditions.[4] With gelatin films, we observed that swelling tends to decrease as gelatin concentration and film thickness increase.

Disintegration of the film matrix results from prolonged swelling time; hence the swelling time was limited to 24 hrs.

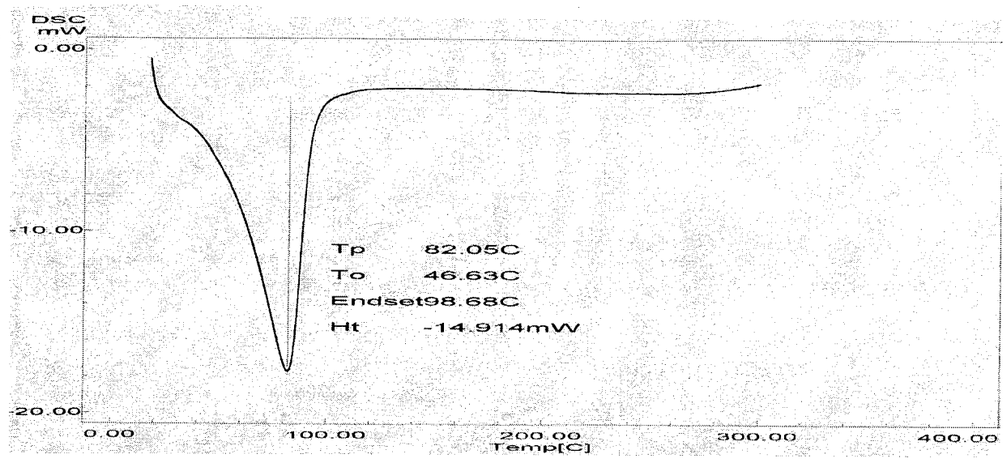
Stability of Films

Film integrity was assessed by differential scanning calorimetry (DSC). The DSC thermograms of the hydrated gelatin films, collagen hydrolysate and crosslinked collagen hydrolysate films are shown in Figure 2 a, b & c, respectively. Comparing Figure 2 a and b, the thermal transition is wider for collagen hydrolysate with an onset temperature of 32.6°C, the endset temperature of 114.3°C and the peak temperature of 86.2°C, with corresponding values of 46.6, 82.1 and 98.7°C, respectively, for gelatin film. This may mean that chrome shaving hydrolysates are more heterogeneous, with the hydrolysates likely varying more in size and molecular weight. The thermogram of the glutaraldehyde crosslinked film (Figure 2c) exhibits a sharp thermal transition, indicating that the structural integrity of collagen hydrolysate film was considerably enhanced by glutaraldehyde crosslinking.

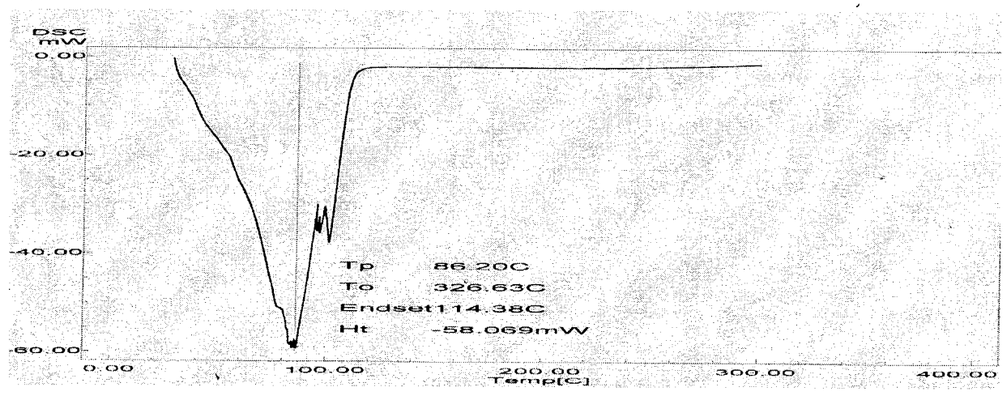
Effect of Crosslinking on Enzyme Immobilization onto Films

The films were crosslinked with glutaraldehyde to increase their structural stability and to provide covalent coupling of enzyme amino groups to collagen. Table I gives the absorbance values proportional to enzyme activity of films crosslinked with 1 and 2% glutaraldehyde, extending for five cycles. In the case of uncrosslinked films, enzyme activity generally decreased with each successive cycle.

a)



b)



c)

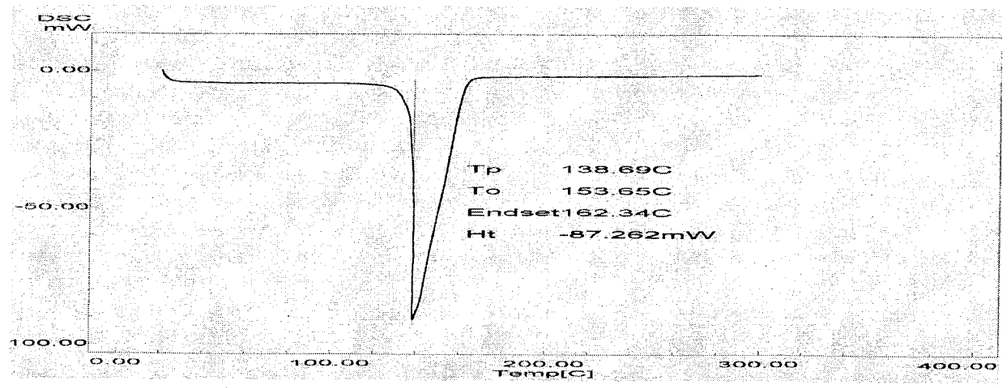


Figure 2. - Differential Scanning Calorimetry for a) hydrated gelatin film, b) hydrated collagen hydrolysate film, and c) crosslinked collagen hydrolysate film

TABLE I

Comparison of Enzymatic Activity from Collagen Hydrolysate Films Cast in Microtiter Plates with and without Crosslinking

Sample No.	Collagen Hydrolysate films	Absorbance at 410 nm Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
1.	Uncrosslinked	0.49	0.29	0.32	0.19	0.14
2.	Crosslinked with 1% glutaraldehyde	0.42	0.38	0.49	0.55	0.57
3.	Crosslinked with 2% glutaraldehyde	0.24	0.37	0.41	0.42	0.45

Table II

Comparison of Enzymatic Activity from Collagen Hydrolysate Films Cast in Microtiter Plates with Enzyme Impregnated before and after Crosslinking

Sample No.	Collagen Hydrolysate films	Absorbance at 410 nm Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
1.	Crosslinked films impregnated with enzymes	0.53	0.51	0.38	0.47	0.43
2.	Films impregnated with enzymes and crosslinked	0.42	0.38	0.49	0.55	0.57

TABLE III

Comparison of Enzyme Activity Yields in Phosphate and Tris Buffers

Sample No.	Type of film	Phosphate buffer pH 7.2 (%)	Tris buffer pH 8.5 (%)
1.	3% commercial gelatin	15.9±0.0	13.4±5.8
2.	6% commercial gelatin	10.9±1.4	8.0±1.5
3.	12% commercial gelatin	7.8±0.9	3.9± 1.0
4.	3% collagen hydrolysate	22.9±1.1	24.2±3.5

Values given are calculated for per gram of dried films

This could be due to enzyme leaching from the films or hydrolytic disintegration of the films. Notice that crosslinked film activity stabilized after the third cycle for both samples 2 and 3. Since, the activity for the 1% crosslinked film was higher; the films were crosslinked to this extent in this work.

Table II gives the absorbance values for enzyme activity of films impregnated with enzymes, before and after crosslinking. Crosslinked films were impregnated with enzymes using glutaraldehyde as the coupling agent. In this series there was a gradual decrease in enzyme activity with the number of cycles. The high initial activity values could be due to loosely bound or unbound interstitial enzyme that might be leached out during cycling. In the case of films crosslinked after enzyme impregnation, the activity became quite stable after the third cycle.

Immobilization of OPH onto Collagen Hydrolysate Films

In general, enzymes were easily immobilized on these films under mild conditions. Since the swelling capacity of the films was about 90%, the enzyme solutions were directly imbibed into the dry films in order to increase enzyme uptake.

The enzyme activity yields in phosphate and Tris buffer are given in Table III. Crosslinked collagen hydrolysate films (in phosphate buffer) yielded the greatest enzyme activities followed by 3% gelatin films. Even though the swelling extent of the 3% gelatin films was marginally higher than that of the collagen hydrolysate films, the decrease in activity of immobilized enzyme could be due to the slight acidity of gelatin compared with collagen hydrolysate. Before casting, the pH of the collagen hydrolysate and gelatin solutions was 7.0 and 5.5, respectively. As the gelatin film thickness increases, the enzyme activity yield decreases. This may be due to the increased diffusion-related difficulties with increasing film thickness.

Reusability of the immobilized enzyme films was assessed by measuring the activity repeatedly over a period of 6 days

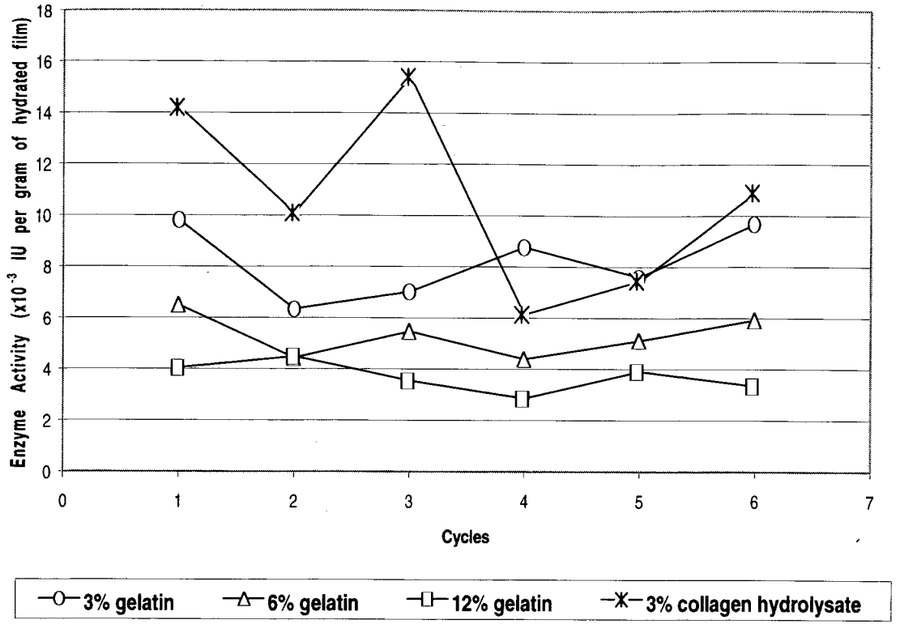


Figure 3. - Activity regeneration of films stored in phosphate buffer

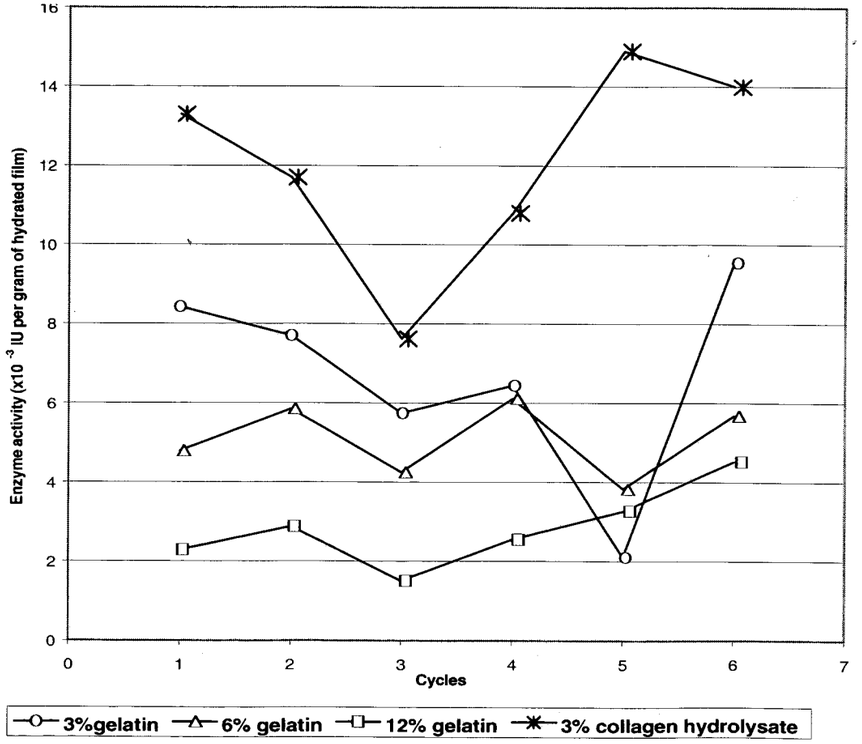


Figure 4. - Activity regeneration of films stored in Tris buffer

at 24 hr. intervals. This 24 hr interval time between cycles of reuse was selected since enzyme activity decreased, if used continuously. The activity retention of immobilized enzymes, stored in phosphate and Tris buffer, is given in Figures 3 and 4, respectively. The activity retention was better in phosphate buffer compared with tris buffer for gelatin, whereas for collagen hydrolysate, the activity retention was found to be better in tris buffer.

CONCLUSIONS

Chrome-containing proteinaceous wastes from the leather industry pose problems when used in landfills due to the theoretical potential for ground water contamination. Recycling by adding value to these wastes cuts overall treatment cost. This study has clearly established the usefulness of the collagen hydrolysate from chrome shavings as a support matrix for immobilization of OPH enzyme. The performance of the collagen hydrolysate films was somewhat better than that of the gelatin films in terms of activity yield and activity regeneration for continued use. pH 8.5 Tris buffer was found to be a better storage medium than pH 7.2 phosphate buffer for activity regeneration. A reliable, inexpensive system for producing sufficient quantities of active preparations of organophosporus hydrolase enzyme was established. These preparations were suitably coupled to the leather-derived, collagenous supports, yielding immobilized degradative enzyme-bearing films. The resulting films showed continuous and recyclable activity for degradation of a model organophosphorus coumpound, Paraoxon.

On the whole, the present investigation opens up new avenues for the use of chrome-containing proteineceous wastes generated in the leather industry. Hydrolysate digest materials can now be effectively used as supports for enzyme-based bioreactors. The need for such cost-effective support matrices is understandable, considering the high production cost of pure forms of the degradative enzymes. Leather-based materials are, in fact, ideal sources of these materials.

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