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Hydrophobic Support: A Phenomenon of Interface Lipase Activation in Polyurethane Foam as a Heterogeneous Biocatalyst in Synthesis of Natural Flavor Ester

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ABSTRAK

Biokatalis heterogen memerlukan penyangga yang sesuai melalui teknik imobilisasi enzim, terutama jika digunakan dalam industri makanan. Dalam sintesis perisa ester alami, busa poliuretan (PUF) dipilih sebagai penyangga imobilisasi lipase, karena memiliki sifat kaku inert, dan porositas tinggi. PUF perlu dilapisi dengan co-immobilized, yang terdiri dari campuran surfaktan yang aman yaitu gelatin, lecithin, PEG, $MgCl_2$, sehingga menjadi satu kesatuan sebagai penyangga PUF hidrofobik. Interaksi hidrofobik antara lipase dan surfaktan pada PUF dapat memicu lipase yang mengaktifkan antarmuka untuk bereaksi lebih banyak dengan substrat melalui sisi aktifnya. Penelitian ini bertujuan untuk mempelajari kemampuan penyerapan PUF pada co-immobilized lipase sebagai biokatalis heterogen. Tahapan yang dilakukan adalah PUF direndam dalam co-immobilized dengan perbandingan 1:10; 1:20; 1:30 (b/b) selama 1-5 jam, kemudian dikeringkan, hasilnya direndam dalam lipase dan dikeringkan, menghasilkan biokatalis heterogen, hasil terbaik biokatalis heterogen diuji pada sintesis perisa ester alami. Hasil penelitian menunjukkan bahwa kondisi penyerapan surfaktan terbaik diperoleh selama 3 jam perendaman pada semua perbandingan PUF: co immobilized 1:10; 1:20; 1:30 (b/b) masing - masing 6,95 g/g; 23,54 g/g; 19,95 g/g, dan aktivitasnya berturut turut 2 U/g PUF; 5,86 U/g PUF; 3,34 U/g PUF. Hasil biokatalis heterogen terbaik pada rasio PUF: co immobilized 1:20 (b/b) diuji pada sintesis perisa alami melalui reaksi esterifikasi asam laurat dari minyak kelapa dan sitronelol dari minyak sereh, menghasilkan konversi 55% perisa alami citronellyl laurat.

***Kata kunci*:** lipase, penyangga hidrofobik, aktivitas antar muka, esterifikasi, perisa alami.

ABSTRACT

Heterogeneous biocatalysts prepared through the enzyme immobilization technique require an appropriate carrier, especially if they are used in the food industry. In the synthesis of natural ester flavor, polyurethane foam (PUF) was chosen as the lipase immobilization carrier, because it has rigid properties, inert, and high porosity. Carrier PUF needs to be coated with a food-safe surfactant known as co-immobilized, consisting of a mixture of gelatin, lecithin, PEG, and $MgCl_2$, so that it becomes a single unit as support for hydrophobic PUF. The interaction hydrophobic between lipases and surfactants in PUF can trigger interface-activating lipases to react more with substrates through their active sites. This study aims to study the sorption capability of PUF on co-immobilized lipase as a heterogeneous biocatalyst. The steps taken were PUF was immersed in co-immobilized in a ratio of 1:10; 1:20; 1:30 (w/w) for 1-5 h, then dried, the results were soaked in lipase and dried, producing heterogeneous biocatalysts, the best results of heterogeneous biocatalysts were tested by natural flavor ester synthesis. The results showed that the best sorption conditions were obtained for 3 hours of immersion in all PUF: immobilized co ratio 1:10; 1:20; 1:30 (w/w) was 6.95 g/g; 23.54 g/g; 19.95 g/g, and each activity was 2 U/gram PUF; 5.86 U/gram PUF; 3.34 U/gram PUF. The best result of heterogeneous biocatalyst at the ratio of PUF: co immobilized 1:20 (w/w) was tested on the synthesis of natural flavors through the esterification reaction of lauric acid from coconut oil and citronellol from citronella oil, resulting in a conversion of 55% to citronellyl laurate natural flavor.

***Keywords*:** lipase, hydrophobic support, interface activating, esterification, natural flavor.

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1. INTRODUCTION

The use of enzymatic technology to synthesize commercial products is becoming increasingly popular. This is due to the environmentally friendly characteristics of enzymatic technology, such as low process energy requirements, high enzyme specificity, eliminating side reactions, and the product's purity, which results in a low by-product. When compared to processes that use inorganic catalysts, this technology uses enzymes as biocatalysts and is more efficient. Immobilization is one of the enzymatic methods used, in which the enzyme is placed in a specific space where it cannot move and can be reused [1–4].

The enzyme immobilization technique was used to increase the free enzyme's stability to pH, temperature, and substrate concentration. This immobilization technique has been widely used in food, beverages, agrochemicals, pharmaceuticals, cosmetics, emulsifiers, flavors, and fragrances as esters. This method employs support as a matrix to bind with enzymes via physical and chemical. The support is attached to the enzyme by adsorption in the physical method, whereas a covalent bond is formed in the chemical method. The covalent immobilization technique works by activating the surface of the support that is linked by functional groups such as aldehydes and glutaricdehydes. Cross-linking is possible between the enzymes themselves [5–7].

Some of the commonly used supports for lipase immobilization are ceramics, silica, various polymers, resins, celite, carbon nanotubes, magnetic particles, and microspheres. Support used in industry is expected to have good physical and chemical properties, including low price, stability to temperature and chemicals, ease to recovery, have a high affinity for lipase, inert, and has an active group [6].

The use of synthetic polymers benefits from the monomer properties of the polymer, namely physicochemical and mechanical properties, which are achieved via active functional groups on the polymer surface. The type of lipase support attachment, physical adsorption, or covalent binding, is determined by this. This polymer's structure includes epoxy, carboxyl, amine, carbonyl, hydroxyl, diol, and alkyl and trialkyl amine active groups [8,9]. The number and type of active groups will provide support, hydrophobic or hydrophilic properties, both of which have the potential to be hydrophobic and have polar interactions with lipases. The use of synthetic polymers has the advantage of allowing the emergence of spacer arms, which extend the enzyme and support bonds, increasing elasticity and avoiding instability due to temperature effects [3,10,11].

PUF, one of the synthetic polymers, can be used as a support for enzyme immobilization in biochemistry and biotechnology. Several researchers have focused on PUF as an inert, rigid, non-toxic material with high porosity, surface area, good hydrophobic/hydrophilic equilibrium, stable performance, and low cost for large capacity use. PUF is a chemically inert material with good mechanical properties. PUF's chemical resistance and high elasticity make it commercially viable due to its low cost. PUF has a high porosity of approximately 97 percent, resulting in a large adsorption surface [6,12,13].

Lipase is an extracellular enzyme with a molecular weight of 31,600 Da, an isoelectric point of 3.8, and a resolution of 1.9 A0. Lipase is made by a variety of microorganisms, including *Mucor miehei*, *Bacillus substilis*, *Thermomyces lanuginosus*, *Candida rugosa*, *Candida cylindracea*, *Aspergillus niger*, *Rhizopus oryzae*, *Penicillium camembert*, and *Penicillium roquefortii* [6,14]. Lipase has two types of confirmation in equilibrium as a homogeneous solution. First, lipase is a close form (inactive) with a helicoidal polypeptide chain structure, flat or lid, with the active side away from the media. Second, a lipase acts as an open form (active), where the lid approaches the hydrophobic surface and the catalytic site of the enzyme is exposed. This

causes more bound substrate so that the reaction rate increases and more products are produced through the active site. It is known that lipid hydrolysis or esterification reactions by lipase are activated by an oil-water interface [12,15].

Lipase immobilization on hydrophobic supports is characterized by the presence of lipase interfacial activation on the support's surface. The percentage of lipase molecules in the monomer and open forms determines the success of this immobilization method. There is no need for an external interface to open the lipase lid as a result of this. The hydrophobic support as heterogeneous biocatalysts can change the final properties of lipase to become more stable, and reusable [6,12,16,17].

The focus of this research is to study the effect of surfactant sorption as co immobilized on the manufacture of heterogeneous PUF biocatalyst. The best results of heterogeneous biocatalyst were tested on the esterification reaction of lauric acid from coconut oil and citronellol from citronella oil-producing natural flavor eater citronellyl laurate. The use of natural flavors is used to reduce the consumption of artificial flavors which cumulatively hurt health. PUF is synthesized from polyols and isocyanate. The performance of PUF supports is activated by a surfactant (co immobilized) as a spacer arm to attach lipase through its interfacial activation ability (Figure 1).

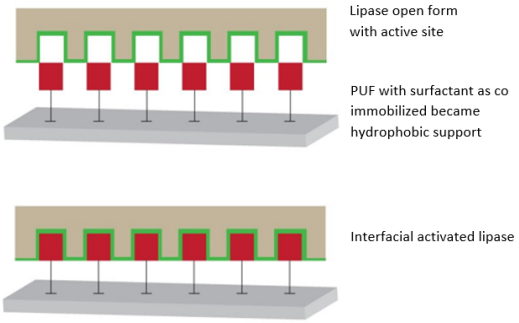

Figure 1. Hydrophobic support PUF arrangement via interfacial activation lipase

The performance of this PUF was analyzed through its sorption support ability to a mixture of surfactants PEG, $MgCl_2$, gelatin, and lecithin, before being attached with lipase. The choice of this surfactant mixture was chosen as an edible ingredient because the flavor product is used for food.

2. MATERIAL DAN METHODS

2.1. MATERIAL

The *Rhizomucor miehei* lipase solution 20,000U/g protein purchased from Sigma Aldrich (St Louis USA) was diluted up to 1,000 x, according to research requirements. Polyols (100 ml)and isocyanate (100 ml) to make PUF are purchased from the Indonesian local market. Coconut oil (purity of 100%) was obtained from local Permata Agrindo Pendowoharjo, Sewon, Bantul, Indonesia with the commercial name Laitco. Co-immobilized consisting of gelatin, lecithin, polyethylene glycol (PEG), and $MgCl_2$ were purchased from Merck (Darmstadt, Germany). Citronellol was isolated from citronella oil from SSE Agroatsiri, Karanganyar, Central Java Indonesia. Bovine Serum Albumin-Sigma Aldrich Science Park Drive 05-01/12 Ascent Building Singapore.

2.2. METHODS

2.2.1. PREPARATIONS OF SURFACTANTS

Make a surfactant mixture of lecithin 50 gL^{-1}, gelatin 50 gL^{-1}, $MgCl_2$ 10 gL^{-1}, and PEG 20 gL^{-1}. Each ingredient is dissolved in distilled water before being mixed with the same weight (200 g) to form a surfactant solution/media [11,18].

2.2.2. LIPASE IMMOBILIZED IN PUF TRANSFORMS INTO HYDROPHOBIC SUPPORT.

In principle, PUF is attached to the co-immobilized/surfactant spacer arm, followed by lipase binding [6,13,19]. PUF (20 g) was cut into 5 mm x 5 mm x 5 mm squares, placed in a glass beaker, and immersed in a surfactant solution to PUF ratio of 1: 10; 1:20; 1:30 (w/w) for 1, 2, 3, 4, 5 hours. The

PUF was then dried in an oven at 30°C for 1 hour. The PUF is then soaked in lipase for 24 hours before being dried in a 30°C oven. PUF is ready for use as hydrophobic support for lipase immobilization via the covalent method between lipase and support and crosslinking between enzymes using surfactants. The experiment was repeated three times. The sorption capacity test was used to evaluate the support of hydrophobic PUF. This method was developed to assess the sorption capacity of surfactants in PUF as a sorbent using ASTM F726-99: Standard Test Method for Adsorbent Sorbent Performance [20]. The saturated hydrophobic support of PUF was transferred and weighed in a weighing bottle. The sorbent's surfactant sorption can be calculated using the equation below.

$$Surfactant\ sorption\left(\frac{g}{g}\right) = \frac{Ss-So}{So} \qquad (1)$$

Where So is the dry sorbent's initial weight and Ss is the sorbent's weight with the adsorbed surfactant.

2.2.3. ASSAY OF LIPASE ACTIVITY

Lipase activity in immobilized PUF was measured using olive oil as a substrate, as modified by previous reports [11,21]. For 2 min, 25 mL of olive oil and 75 mL of 7% gum arabic solution were emulsified. The emulsified olive oil was then combined with 10 ml of 0.1 M phosphate buffer (pH 7) and 1 gram of immobilized PUF in a 25 ml volume. For 30 minutes, the mixture was incubated at 37°C with an orbital shaker. Following incubation, the reaction was stopped by adding 15 ml of acetone-ethanol (1:1 v/v), and the free fatty acids were titrated with 0.05 M NaOH, using phenolphthalein as an indicator (pp). One unit of lipase activity was defined as the amount of enzyme capable of releasing one mol of fatty acid per minute. Equation (2) can be used to calculate the lipase activity on the PUF support.

$$Lipase\ activity\ (U/\text{gram}) = (Vs - Vb)x\ N\ NaOH\ x\ 1000\ /t \qquad (2)$$

where Vs and Vb are the volumes of NaOH (ml) required to titrate the sample and the blank.

Heterogeneous biocatalyst samples PUF, PUF-co-immobilized, PUF-lipase, PUF-co-immobilized-lipase, were analyzed for their surface structure morphology. The instrument used was SEM-JEOL JSM-7800F (Pleasanton, California, United States).

2.2.4. ESTERIFICATION

The best results from the sorption of surfactants in PUF as a heterogeneous biocatalyst were tested on synthetic natural flavors through the esterification reaction of lauric acid hydrolyzed coconut oil and citronellol from citronellal oil isolation. The lauric acid used accounted for 1.07 percent of the free fatty acids produced by hydrolysis of coconut oil, or 0.0003 mol. Citronellol was also used in the amount of 0.0003 mol or 0.05 grams. The esterification reaction was carried out in an Erlenmeyer flask for 20 hours at 40°C in a shaker incubator at 120 rpm. The reaction was stopped by adding 10 mL of acetone-ethanol (1:1 v/v) mixture [21]. There are two layers, with the natural flavor of the citronellyl laurate ester on top and water on the bottom. The top layer was subjected to analysis. Citronellol conversion was obtained from the analysis of initial and after esterification reaction using GC FID Thermo Fisher, type column r stabilizer wax - DA (30 m x 0.25 mm x 0.25 m, polyethylene), oven temperature 75°C -200°C.

3. RESULTS AND DISCUSSION

3.1. SEM ANALYSIS

The surface structure of blank PUF cubes and modified PUF with surfactants, lipases, and surfactants plus lipase can be seen in Figures 2a,b,c,d. Figure 2a shows that the cell structure of the blank PUF cubes is not ideal as a pentagonal dodecahedron, but rather as an open-cell caused by CO_2 gas bubbles formed by the reaction of polyols and isocyanates [13,20,22]. In Figures 2 b, c, the addition of surfactant/lipase gave a different surface compared to a smoother PUF blank.

Lipases through their covalent bonds can attach to the porous PUF surface. In Figure 2d the addition of surfactant as co-immobilized lipase consisting of lecithin, gelatin, PEG, and $MgCl_2$, it can be seen that there is an agglomeration of these materials. Lipase attaches to the surface of the PUF which has been coated with a surfactant as a spacer arm. This covalent bond interaction is very compatible to attach PUF with lipase to become strong hydrophobic support as a heterogeneous biocatalyst. The same phenomenon can be seen in SEM lipase *C Antarctica B* immobilized using PUF in the synthesis of geranyl propionate and ethyl oleate [20,22]. The surface of blank PUF is smoother than the surface of PUF that immobilizes lipase Y lipolytica by coating polyethyleneimine (PEI) and glutaraldehyde (GA) coupling. The surface of blank PUF is smoother than the surface of PUF that immobilizes lipase *Y lipolytic* via PEI and GA coupling coating [13].

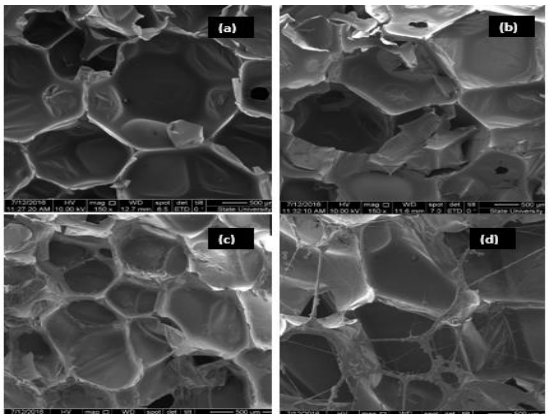

Figure 2. SEM of (a) PUF, (b) surfactant coated PUF, (c) lipase attached PUF, (d) surfactant coated PUF, and lipase attached magnitude 300x

3.2. SURFACTANT SORPTION CAPACITY

Immersion of the PUF blank in the surfactant is used to improve the support performance to become hydrophobic. The natural flavor esterification reaction requires a hydrophobic heterogeneous biocatalyst because the substrate is lipid.

The porous characteristic of PUF blanks provides opportunities for penetration of surfactant solutions.

The length of immersion time affects the lipase loading on the support, depending on the surfactant sorption capacity [20].

Surfactant sorption capacity is a method used to determine the absorption ability of PUF on surfactants before being attached to lipase. The surfactant sorption capacity (g surfactant/g PUF) determines the enzyme loading on the hydrophobic support, expressed in U/gram PUF. Blank PUF cubes had a macrospore cell size between 90 – 200 µm modified with surfactant coating [20].

Figure 3a. showed the same trend of surfactant sorption velocity for all PUF: surfactant ratios at 1 hour to 5 hours immersion. The highest surfactant sorption velocity was obtained at 1-hour immersion, then it decreased until 5 hours. The surfactant filled the PUF blank pores with the highest speed in the first 1 hour for each ratio of PUF: surfactant 1:20; 1:30; 1: 10 (w/w), each sorption velocity; 40; 28.6; 19.7 (w/w) then at 5 hours decreased to 9.16; 6.2; 3.3 (w/w). This decrease was caused by the very fast penetration of surfactants into the empty pores of PUF at the beginning. But with increasing time with the same and the same amount of surfactant load, the sorption velocity decreased.

Figure 3b shows the trend of surfactant sorption in all PUF: surfactant ratios indicate that 3 hours of immersion time is ideal for the PUF macrospore cell structure. Surfactant sorption at a ratio of PUF: surfactant 1:20 (w/w), having the highest value of 23.54 g/g PUF compared to ratios of 1: 10 (w/w) and 1: 30 (w/w), respectively. has a value of 6.95 g/g PUF, and 19.95 g/g PUF, for 3 hours of immersion. The low surfactant sorption capacity at a ratio of 1: 10 (w/w), indicates that the amount of microemulsion surfactant slightly affects the amount of surfactant absorbed. Whereas after 3 hours of immersion, the ratio of PUF: surfactant 1: 30 (w/w), surfactant sorption decreased drastically by 37% unlike 1: 10 (w/w) and 1:

20 (w/w) were more stable. This shows that at a ratio of PUF: surfactant 1: 30 (w/w) amount of microemulsion surfactant is too much which cannot be absorbed by PUF [13,24-25]. This is due to the saturated surfactants and high water content causing the fractured PUF structure to be less rigid (physically visible). The best condition at 3 hours of immersion has the ratio of PUF: surfactant 1:20 (w/w), due to the synergy of surface activity of gelatin and lecithin on hydrophobic support to form an effective complex to avoid particle aggregation [23].

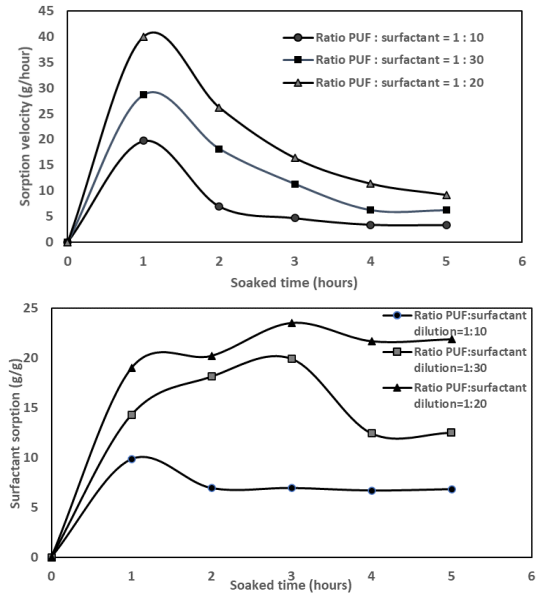

Figure 3. Sorption of surfactant on PUF as hydrophobic support. a) sorption velocity of surfactant gelatin, lecithin, $MgCl_2$, PEG. b) surfactant sorption on PUF

3.3. INTERFACIAL ACTIVATION OF LIPASE

Modification of PUF blanks through surfactant coating is one of the applications of lipase immobilization technology on the support and will turn into PUF with a hydrophobic character because surfactants have hydrophilic and hydrophobic groups. Immobilized lipase on PUF is more stable than free lipase in the enzymatic synthesis reaction. In this study, the effect of coating using a simple method by immersing PUF in surfactant was studied.

Figure 4, shows a relationship that is close to proportional to Figure 3b. Figure 4 shows, at 3 hours of immersion, the ratio of PUF: surfactant 1:20 (w/w), indicating that the lipase attached to the PUF was higher than the other ratios, which was 4.01 U/g PUF. Unit (U) is the quantity of lipase that will liberate 1 µmol fatty acid per minute While the ratio of PUF: surfactant 1:10 (w/w); 1: 30 (w/w) 2.32 U/g PUF each; 3.34 U/g PUF. Figure 3b shows that the highest surfactant sorption obtained at an immersion time of 1: 20 (w/w) was 23.54 g/g PUF. This shows that every gram of PUF will be able to absorb 23.54 grams of surfactant as a spacer arm and successfully attach 4.01 U of lipase. While the ratios of 1: 10 (w/w) and 1: 30 (w/w), each has a value of 6.95 g. /g PUF, 19.95 g/g PUF. This shows that 6.95 g of surfactant is capable of attaching 2.32 U lipase, and 19.95 g of surfactant can attach 3.34 U lipase. Figure 5 depicts the amount of surfactant absorbed (Figure 3b) as a linear profile of lipase activity immobilized in PUF (Figure 4), with R^2 = 96 % This demonstrates that lipases, as interfacial enzymes, act on the water-lipid interface, particularly on suitable surfactant molecules. Surfactants have hydrophilic and hydrophobic parts, with the hydrophobic part able to activate the PUF support until it becomes hydrophobic, allowing the lipase to interact strongly with the surfactant [6,24,25].

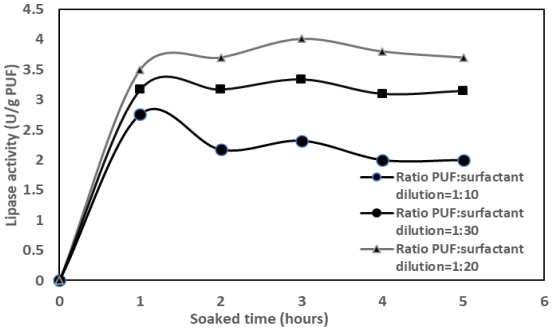

Figure 4. The relationship between lipase activity and soaked time

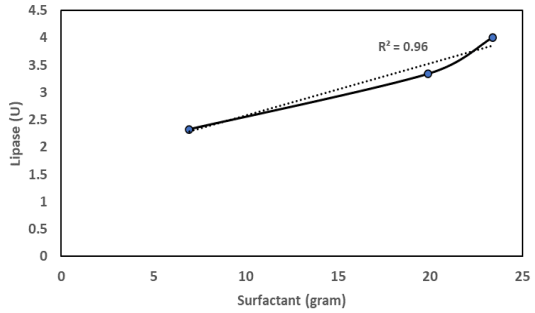

Figure 5. The relationship between the amount of surfactant and lipase activity, at a time of 3 hours of soaked for 1 gram of PUF

3.4. ESTERIFICATION

The best heterogeneous biocatalyst (PUF ratio: surfactant 1:20 (w/w) produced through the interface mechanism of lipase activation in PUF with surfactant coating can be applied for enzymatic synthesis. Lauric acid and citronellol as substrates were reacted under mild conditions at 40°C using immobilized lipase in PUF into natural flavor esters of citronellyl lauric. Initial and final conditions were measured for citronellol using GC FID. The results of this study showed 55% citronellol conversion, this indicates that the heterogeneous biocatalyst of immobilized lipase in PUF can work well.

4. CONCLUSION

Heterogeneous biocatalysts can be obtained by coating edible surfactants to be applied to enzymatic reactions in the food industry. Natural flavors can be synthesized from natural ingredients by enzymatic reactions, using non-toxic heterogeneous biocatalysts, supporting PUF to become "greener" processes.

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