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효소 고정화막의 응용에 대한 총설

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Applications of Enzyme Immobilized Membranes: A Review

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요 약: 생체 내 변화에서 효소는 중요한 촉매이다. 효소의 안정성과 재사용성은 촉매 과정에서 중요한 요소이다. 적합한 기질에 효소 고정화는 특정 미세환경의 조성을 통해 효소 활동성을 높인다. 다양한 종류의 분리막이 각각의 생체적합성과 막 표면의 친수성/소수성 조절 용이도에 따라 기질로 사용되었다. 본 논문에서는 셀룰로스, 폴리아크릴로니트릴(PAN), 폴리디메틸실록산(PDMS), 폴리비닐리덴플루오라이드(PVDF), 폴리에테르설폰(PES) 고분자 분리막이 소개되고 토의되었다. 고정화 효소를 이용한 유기오염물의 생물적 분해는 제약 회사 및 섬유 회사 등에서 발생하는 오염물질을 친환경적으로 감소할 수 있는 방법이다. 효소 고정화 생물반응기(EMBR)로 기름의 가수분해를 제어할 수 있고 이를 통해 탄소 배출량 감소 및 환경오염을 줄일 수 있다. EMBR로 만들 수 있는 바이오에탄올과 바이오디젤은 화석 연료의 대체제이다.

Abstract: Enzymes are important class of catalyst for biotransformation. Stability and reusability of enzymes during the catalysis process is a key issue. Activity of enzyme can be enhanced by its immobilization on a suitable substrate by creation of specific microenvironment. A variety of membranes has been used as substrate due to the biocompatibility and simpler method to tune hydrophilicity/hydrophobicity property of the membrane surface. In this review, polymer membranes including cellulose, polyacrylonitrile (PAN), polydimethylsiloxane (PDMS), polyvinylidene fluoride (PVDF), polyethersulfone (PES) are introduced and discussed in detail. Biodegradation of organic contaminants by immobilized enzyme is an environmental friendly process to reduce the contamination of environment in pharmaceutical company and textile industries. The controlled hydrolysis of oil can be performed in enzyme immobilized membrane bioreactor (EMBR), resulting in reducing carbon emission and reduced environmental pollution. Bioethanol and biodiesel are considered alternative fossil fuels that can be prepared in EMBR.

Keywords: *enzyme immobilized membrane bioreactor, bioethanol, biodiesel, bio degradation*

1. Introduction

Environmental pollution due to continuous increase of fossil fuel leads to finding out alternative source of energy. Bio-based fossil fuel are alternative source of energy. Enzymes, present in living system are known

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as biocatalyst[1-5]. Advantage of these over chemical catalyst is that they are i) environmental friendly leading to ease of disposal, ii) highly selective resulting in pure product, iii) reactive under mild condition and iv) abundantly availability. Enzymes are classified as oxido-reductases, transferases, hydrolyases, lyases, Isomerases and ligases.

Recycling of catalyst is very important in any chemical process. Due to high sensitivity to temperature, pH and sensitivity towards solvent, it restricts reuse of enzyme. Although there is abundant availability of enzyme but their isolation and purification are very expensive. Enzyme immobilization is a special technique that enhances bioactivity and can be recycled for several times, thus reducing the cost of the chemical transformation process[6-10]. Concentration of enzyme immobilization is based on the surface area of membrane. It has comparatively much more efficiency than catalysis based on free enzyme. *Saccharomyces cerevisiae* cells are immobilized on suitable membrane for production of bioethanol. Similarly, *lipase* based enzymatic membrane reactor (EMR) are used for generation of bioethanol.

Laccase is another class of ligninolytic enzymes that oxidize organic molecules nonspecifically with a lot of scope in wastewater treatment. Pharmaceutically active compounds present in wastewater are degraded by laccase immobilized membrane reactor[11-13]. This review is classified mainly into biodegradation, bioethanol, and biodiesel production by EMR which is mainly based on several polymer membranes including cellulose, polyacrylonitrile (PAN), polydimethylsiloxane (PDMS), polyvinylidene fluoride (PVDF), polyethersulfone (PES). The schematic representation of classification and polymers used in this review is shown in Fig. 1 and summarized in Table 1.

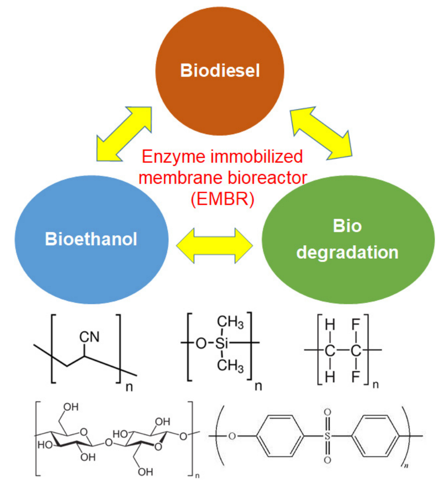

Fig. 1. Schematic representation of classification and polymers used in this review.

2. Biodegradation

For effective usage of biphasic enzyme-immobilized membrane reactors (EMBR), loading of enzyme and its continued activity are necessary[14]. A method to produce ultrafine fiber membrane in relation to lipase, meaningfully satisfying the aforementioned requirements have been found. A cellulose acetate ultrafine fiber membrane that is non-woven (200 nm fiber diameter) went through alkaline hydrolysis to get regenerated cellulose (RC). By exposing the RC ultrafine membrane to $NaIO_4$, the membrane has been oxidized, forming entity of aldehyde groups and pentaethylenehexamine (PEHA), which functions as a spacer for immobilization of lipase. The inclusion of spacer increased the retention in activity. The assembly of EMBR with PEHA and lipase immobilized membranes showed high bioreactor activity in the hydrolysis of olive oil under optimum conditions. In another study, a method to increase the loading of enzyme and activity retention of biphasic cellulose membrane with immobilized lipase has been studied[15]. The bioreactor was first composed with spiral form wound cellulose nanofiber membrane by electrospun. Alkaline hydrolysis, oxidation using $NaIO_4$ and pentaehthylenehexamine modification was performed on the support to increase the efficiency of the catalyst. After the modification, lipase was covalently immobilized. Experiments show 44.3% activity retention and 28.9 mg/g enzyme loading with the immobilization of lipase. Variables such as flow rate in organic phase, aqueous phase as well as concentration of substrate were varied to study their ef-

Table 1. Summary of Enzyme Activity for Various Membranes

Membrane	Enzyme	Reactor	Activity	Application	Reference
CA	Lipase	EMBR	Oil hydrolysis	Biodegradation	[14]
Cellulose nanofiber membrane	Lipase		Oil hydrolysis	Biodegradation	[15]
PAN-co-acrylic acid nanofiber	CRL	Biphaseic lipase-immobilized nanofiber	Oil hydrolysis	Biodegradation	[16]
				Biodegradation	[17]
PAN-biochar	Laccase		Pharmaceutical compound degradation	Biodegradation	[18]
	Microalgae	PBR	Microalgae cultivation, harvest, membrane fouling control	Biodegradation	[19]
PMMA CEA	HRP	Polyethylenimine, glutaraldehyde	Bisphenol A Removal	Biodegradation	[20]
PAN/PVdF EFMs	Laccase		Pollutant removal	Biodegradation	[21]
PVA/polyacrylic acid/SiO_2	HRP		Paracetamol removal	Biodegradation	[22]
PVA/CS/MWNTs	Laccase			Biodegradation	[23]
Silicalite-1/PDMS/PVDF		SSB	Ethanol fermentation-pervaporation integration	Bioethanol	[24]
Calcium alginate film, Silicalite-1/PDMS	Invertase		Bioethanol production / purification	Bioethanol	[25]
PES	CRL	NC-SiO_2	Esterification	Biodiesel	[26]
PAN	Lipase		Conversion of biomass	Biodiesel	[27]
Fe_3O_4@SiO_2 Nanocomposite	CRL			Bio-catalytic processes	[28]
PES	CRL	EHMR		Biodiesel	[29]
		MBR	Anammox process operation	Mitigate MBR membrane fouling	[30]

CA: cellulose acetate; EMBR: Enzyme-immobilized membrane reactors; PAN: Polyacrylonitrile; PVdF: Polyvinylidene fluoride; EFM: Electrospun fibrous membranes; PBR: Photobioreactor
HRP: Horseradish peroxidase; PMMA CEA (Polymethyl methacrytate-co-ethyl acrylate; PVA; Polyvinyl alcohol; CS: Chitosan; MWNT: Multi-walled carbon nanotubes; PDMS: Polydimethylsiloxane; SSB: Sweet sorghum bagasse; PES: Polyethersulfone; CRL: *Candida rugosa* lipase; NC: Nanocellulose; EHMR: Enzymatic hybrid membrane reactors; MBR: Membrane bioreactor

fects on the performance of the bioreactor. The result using hydrolysis of olive oil showed that pure olive oil substrate had a 100% hydrolysis conversion after 9 organic circulations with 10.5 mL/min and 600 mL/min flow rate for organic phase and aqueous phase respectively. These values suggest for the usage of these bioreactors in lipase-catalyzing reactions. The characteristics of biphasic PANCAA nanofiber membrane with covalent immobilization of CRL has been also studied based on the reaction of olive oil hydrolysis[16]. With the size of the membrane and the parameters of different operation being optimized, the hybrid membrane showed high bioreactor reactivity and operation stability for more than six reactions. The improved reusability and stability suggest the successful usage for industrial usage. The results of the experiment also showed that there exists a balance when optimizing the parameters of membrane and operation. This understanding could

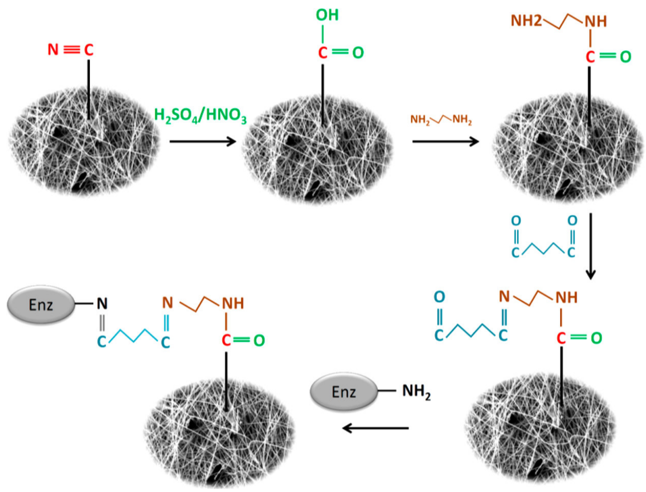

Fig. 2. Laccase immobilized on PAN-biochar nanofibrous membrane (Reproduced with permission from Teheran *et al.*, 31, Copyright 2017, American Chemical Society).

contribute to discovering the method of optimizing the utility of different enzyme immobilizations in its practical usage.

The constant discarding of antibiotics is causing environmental problems[17]. Degradation of these pharmaceutical products using immobilized laccase on polyacrylonitrile-biochar nanofibrous membrane has been studied. Chlortertracycline, a common antibiotic has been used for the experiment. The immobilized enzyme showed better stability in terms of storage, temperature and pH. It also had high reusability, maintaining over 50% of its functionality after 7 ABTS oxidation cycles. Immobilized laccase also showed high removal efficiency for different flux rates, overall, being an effective measure to degrade medical drugs and treat waste water.

Degradation using laccase is an eco-friendly method for removing pharmaceutical compounds disposed in wastewater and the environment[18]. However, its low reusability and stability hinder its industrial usage. Thus, the degradation has been optimized by the covalent immobilization of laccase onto polyacrtlonitrile-biochar nanofibrous membrane via electrospinning, as shown in Fig. 2. The hybrid biocatalyst has been used to remove three main medical drugs, antibiotics (chlortetracycline, CTC), antidepressant (carbamazepine, CBZ) and anti-inflammatory (diclofenac, DCF). According to the investigation, the immobilization of enzyme showed enhancement in storage, temperature and pH stability as well as reusability. The membrane had more than 17% of its initial performance after 10 ABTS oxidation cycles as well as showed high degradation efficiencies for all three drugs after 8 hours of reaction. The decreasing extent of adsorption also confirms the regeneration of laccase on the adsorption sites on biochar.

A photobioreactor (PBR) and ultrafiltration system has been coupled for cultivation and harvesting of microalgae as well as for control of membrane fouling in purifying of anaerobic digestion effluents[19]. The start-up of PBRs with suspended *C. vulgaris* (SCV), immobilized CV (ICV) and ICV with powdered activated carbon (ICV+PAC) have been studies. The results showed that there were high degradation of DOCs and attaining of nutrient removals in PBRs. The consortium of bacteria and microalgae improved the biodegradation. Adsorption of PAC sped up the photodegradation. Immobilized microalgae beads also maintained the integrity of the cell by less debris and breakdown of organic matters in and out of cells. Research also showed that layers of ICV+PAC can trap the leftover pollutants, further controlling the fouling of membrane. In a nutshell, purification of ADE and prevention of membrane fouling can be done by the harvesting of microalgae using ICV+PAC.

The covalent immobilization of horseradish peroxidase (HRP), an enzyme in the roots of horseradish, on polymethyl methacrylate-co-ethyl acrylate (PMMA CEA) microfibrous membranes showed significance in removing bisphenol A from water[20]. While the immobilized maintained 70% of free enzyme's enzyme activity, it had significant improvements in its stability and reusability. The immobilized enzyme still showed performance of 50% of the initial performance, even after being reused for six times. The enzyme also showed enhanced efficiency in removing bisphenol A in 3 hours (93%) than free enzyme (61%) or the membrane on itself (42%). The adsorption of particles on PMMA CEA as well as better catalytic activity of the HPR after immobilization reasons the advancement in the removal of BPA.

Nano-coppers were synthesized with the enzyme carrier polyacrylonitrile/polyvinylidene fluoride (PAN/PVdF) electrospun fibrous membranes (EFMs)[21]. PAN/PVdF/Cu EFMs immobilized with laccase was used to remove 2,4,6-trichlorophenol from water. The physical and electrochemical properties of the hybrid membrane and the efficiency of immobilizing laccase as well as removing 2,4,6-TCP were closely examined. The diameters of the membranes differed, smallest being 200 nm and the largest being 500 nm. The immobilized enzyme showed better durability, pH and thermal stability, as well as removal of 2,4,5-TCP than without the nano-copper. This is due to the facilitation of electron transfer by the nano-coppers during its reaction with laccase. Laccase immobilized PAN/PVdF/Cu EFMs have high reusability, making it a desirable choice for industrial usage when removing pollutants.

While paracetamol is a commonly used medical drug, its accumulation threatens the aquatic environment[22]. Therefore, a study to treat and remove paracetamol from water by horseradish peroxidase and nanofibrous membrane has been conducted. By thermal cross-linking process, polyvinyl alcohol/polyacrylic acid/SiO_2 membrane was made insoluble. HRP was then immobilized using covalent bonding. Although the immobilized enzyme showed 79.4% of the initial activity of the free enzyme, it showed better stability for storage, pH and temperature. It also showed similar performance in removal of paracetamol with free enzyme, with greater reusability. These characteristics make the hybrid membrane favorable for treating wastewater.

The stability and catalytic activity of laccase-immobilized PVA/CS/MWNT nanofibrous membrane for degradation of diclofenac have been investigated[23]. The new membrane had better performance than the conventional membranes by its high porosity, better permeability, as well as greater surface area. The composition of MWNT to the membrane showed significant increase in loading of enzyme and activity retention. MWNTs also contributed to the electrochemical capacitance of the membrane. Immobilized laccase also enhanced the stability, reusability and removal efficiency of the membrane. Based on the study, it can be inferred that carbon nanotubes can improve the transfer of electron between enzymes and substrate, contributing to improved activity of the immobilized enzyme, suggesting a promising method in eliminating pollutants.

3. Bioethanol

A silicalite-1/polydimethylsiloxane/polyvinylidene fluoride membrane effectively carried out the ethanol fermentation-pervaporation integration[24]. The new fermentation system with sweet sorghum bagasse immobilized showed increased productivity of ethanol with the usage of pervaporation. In fed-batch fermentation-pervaporation integration, the productivity of ethanol, separation factor, net flux and permeate ethanol concentration 1.6 g/L h, 8.2~9.9, 319~416 g/m^2 h and 426.9~597.2 g/L, respectively, while that for continuous fermentation-pervaporation were 1.6 g/L h, 7.8~9.8, 227.8-395 g/m^2 h and 410.9~608.1 g/L. The SSB carrier resulted in enhanced alcohol productivity and lower concentrations of cells that are suspended. The membrane made the system useful in long operations. The outcomes suggest for the improvement in production of ethanol and its separation with the usage of the new integration process.

Saccharomyces cerevisiae-immobilized calcium alginate film has been used for the anaerobic breakdown of sugar to produce ethanol[25]. Microchannel surface with calcium alginate was used in the process. Alginate films with yeast immobilization showed better yield of ethanol than free yeast, given the same condition. For the separation of ethanol, a composite membrane with 3-1 ratio of silicalite-1 to PDMS was found to be the most efficient. These two outcomes suggest for possible application of integrated reaction-separation measure for producing and purifying bioethanol.

4. Biodiesel

An eco-friendly esterification technique for usage in biofuel (pentyl valerate, PeVa) production has been studied by optimizing the covalent immobilization of the enzyme *Candida rugosa* (CRL) to a polyethersulfone (PES) membrane reinforced by nanocellulose-silica (NC-SiO_2) composed by biological materials[26]. Meaningful conjugation of the CRL to NC-SiO_2-PES has been verified using Raman spectroscopy, field emission scanning electron microscopy, high-resolution transmission electron microscopy and atomic force microscopy of the hybrid entity. The most efficient method to synthesize PeVa is by immobilizing CRL with a concentration of 20 mg/mL for 4 hours, stirred at a 40 °C, pH5 environment with 5% glutaraldehyde. This creates an entity which is up to 30% more stable than free enzyme at the same temperature and reusable for a maximum of 14 esterification reactions. These results show that the immobilization of CRL onto NC-SiO_2-PES membrane is a promising method for esterification of PeVa and opens ground for further research in improving the performance of CRL in terms of its durability and activity by using NC and SiO_2 to fill PES.

Immobilization of lipase in the production of biodiesel using enzymes has shown to have various benefits[27]. PAN-PEI-SA-$CaCL_2$ membrane was synthesized for immobilization of lipase. The loading of protein and the enzyme activity both showed significant enhancements, resulting in high biodiesel yield (78.5%). The immobilized enzyme also showed how reusability, losing only 11% of its original performance even after usage for over 20 times. The results of this experiment suggest the application for converting biomass resources. A study on using other groups to reduce the detachment of enzyme for reusing would be further the benefits of this process.

5. Others

Membrane-immobilized enzymes have high potential in being a useful part in the field of biosensors, enzymatic reactors and membrane bioreactors[28]. The effect of covalent immobilization of CRL on nanocomposite membrane has been studied. Fe_3O_4@SiO_2 nano-particles were coated on the surface of ultrafiltration membrane using the LTH process, followed by reaction with 3-APTS. Glutaraldehyde functioned as a coupling agent for the immobilization of lipase on the membrane surface. Various tests such as SEM and TEM evidenced success of immobilization on its efficiency and the activity of enzyme. The nanocomposite membrane contributed to enhanced activity and loading capacity. According to kinetic parameters of the reaction, the increase in substrate affinity and decrease of enzyme catalytic activity both decreased. Based on its better thermal, storage and operational stability, the immobilized enzyme is a favorable subject for bio-catalytic process.

The reusability, stability and cost-effectiveness to separate compounds make membrane-immobilized enzyme an attractive bioreactor for further exploration, especially for production of biodiesel and biosensing [29]. The formation and hydrophilicity are often modified to adapt the membrane for enzyme immobilization. Different percentages of graphene oxide nanosheets (GON, 0.00, 0.25, 0.50, 1.00, 2.00 and 3.00) have been synthesized to the nanocomposite polyethersulfone (PES) membrane via phase inversion method. *Candida rugosa* lipase (CRL) also supply enzymatic hybrid membrane reactors (EHMRs) to the membrane. The effect of enzyme immobilization on membranes with different amounts of GON has been tested by pure water flux. The results show that the more the GON, the greater the hydrophilicity. The experiments show that

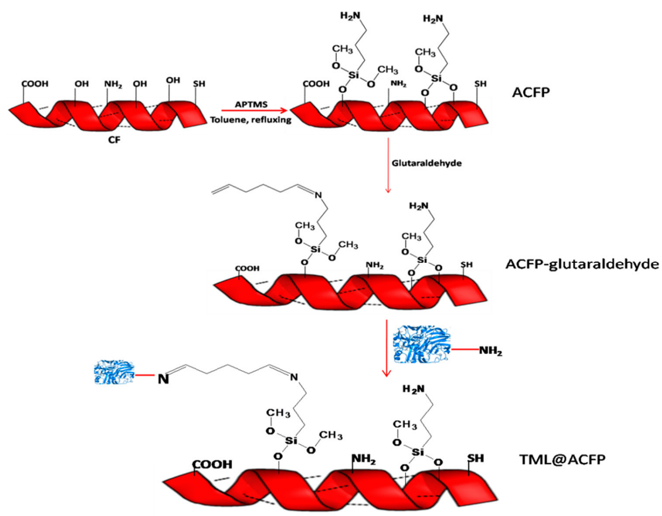

Fig. 3. Schematic diagram of the immobilization course of TML on ACFP (Reproduced with permission from Suman *et al.*, 31, Copyright 2019, American Chemical Society).

membrane with 1.00% GON is the best in terms of its activity, reusability and permeability and has better stability for both pH and temperature compared to both free and immobilized CRL. In general, wet hybrid membrane showed better stability than dry membrane, and those kept at 4 °C were more stable than at room temperature.

The effect of the existence of magnetic porous carbon microsphere carriers on anammox MBR fouling membranes has been studied[30]. According to the research, trans membrane pressure showed significant decrease after 50 days due to the use of carriers in indirect contact with the membrane. The SEM results show that anammox bacteria formed a film on the surface of the membrane, hindering direct contact. The membrane surface is more hydrophobic due to the metabolite discharged by anammox bacteria; the metabolite has more of hydrophobic groups. The immobilization is beneficial as it lowers the fouling of biological membrane and organic matter that contains carbonyl hydrophilic groups, as well as deposition of inorganic substances. These suggest a measure to stop the fouling of MBR membrane in anammox process.

Chicken feather was amino-functionalized and 74.24 % of Trametes maxima laccase (TML) was immobilized onto it[31]. Half life time, decimal reduction time, Gibbs free energy and change in enthalpy of the

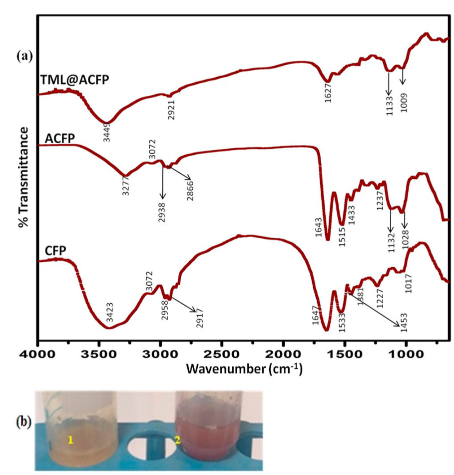

Fig. 4. (a) FT-IR spectra of chicken feather powder (CFP), amino-functionalized chicken feather powder (ACFP), and immobilized TML on ACFP TML@ACFP. (b) Evidence of amino functionalization: (left) chicken feather without amino functionalization and reaction with glutaraldehyde; (right) amino-functionalized chicken feather after reaction with glutaraldehyde. Change of color indicates the Schiff base formation (Reproduced with permission from Suman *et al.*, 31, Copyright 2019, American Chemical Society).

immobilized enzyme were enhanced as compared to free enzymes. It can be stored at higher temperature and pH, indicating increment in the stability. Enzyme catalyzed oxidation of non-phenolic compounds were studied. Fig. 3 and 4 represent schematic representation of amino-functionalization on ACFP and its FTIR char-

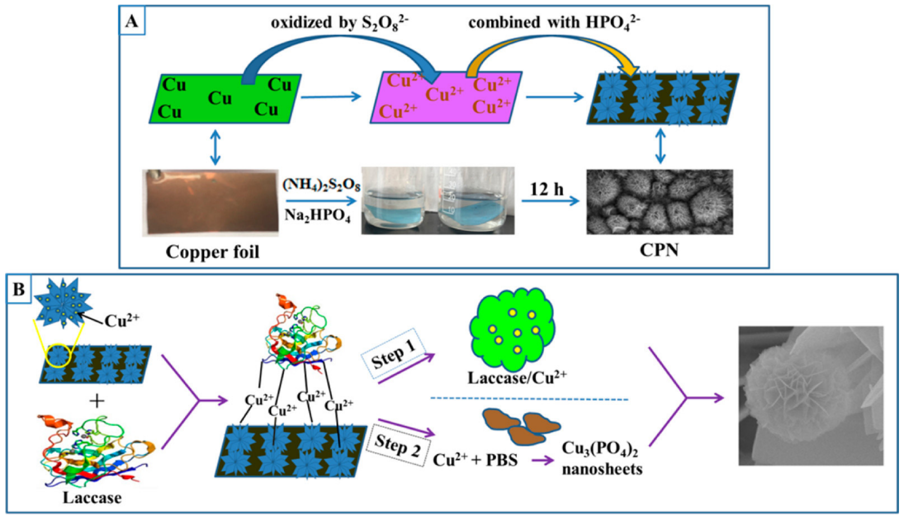

Fig. 5. Schematic diagram of the formation mechanisms of (A) CPN and (B) the La–CPN hybrid material (Reproduced with permission from Rong *et al.*, 32, Copyright 2017, American Chemical Society).

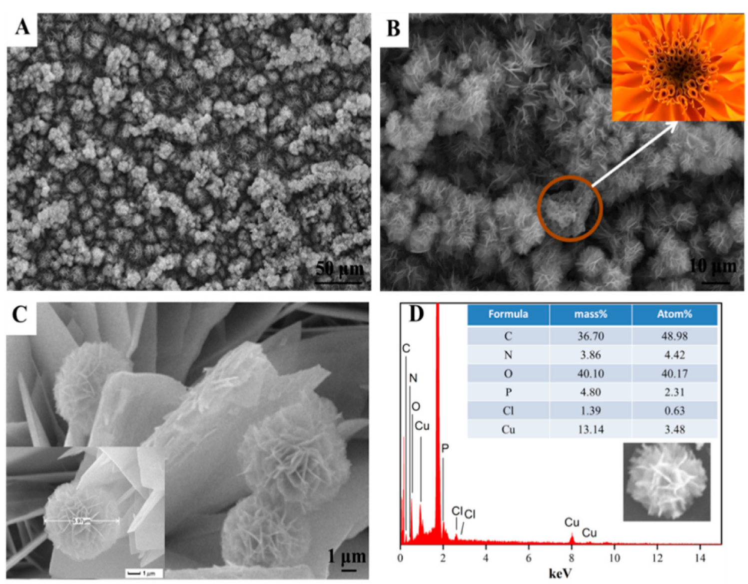

Fig. 6. (A–C) SEM images under different magnifications and (D) EDS spectrum of La–CPN hybrid nanoflowers. The inset in (B) makes the analogy to a pistil more evident, showing the growth process of the nanoflowers. (Reproduced with permission from Rong *et al.*, 32, Copyright 2017, American Chemical Society).

acterization, respectively.

Enzymatic degradation of organic contaminants is critical to the control environmental pollution. Stability and recycling related issue can be improved by immobilization on substrate. The researchers immobilized laccase-$Cu_3(PO_4)_2$ hybrid on $Cu_8(PO_3OH)_2(PO_4)_4 \cdot 7H_2O$ nanoflowers grown on copper foil[32]. Organic dye such as Congo red dye is an effluents of textile industry and can be degraded more than 95% by this immobilized enzyme. At the same time, the rate of degradation is about 3.5 times higher than the free laccase enzyme. The oxidation ability of the enzyme is catalyzed by the presence of copper ion. The schematic figure of degradation process and morphology of the substrate for enzyme immobilization are represented in Fig. 5 and 6, respectively.

6. Conclusions

Enzyme immobilized membrane reactor is an efficient technique for various chemical transformation compared to free enzyme catalysis. Pharmaceutically active compounds present in pharmaceutical industry effluents can be degraded by laccase based EMR. In order to replace fossil fuel with alternative source of energy, bioethanol and biodiesel are considered future fuels. These fuels can be generated by EMBR by immobilizing lipase. Membrane with different shape such as flat sheet, hollow fiber and nanofiber membrane are used as the support for enzyme. Higher surface area and suitable functional groups present on the membrane surface enhanced the concentration of the immobilized enzyme. In this review, different types of membrane support are introduced and discussed for use in EMR for applications in drugs degradation and alternative fuel production.

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