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DOI
10.1021/acssuschemeng.9b01075

Publication date
2019

Document Version
Final published version

Published in
ACS Sustainable Chemistry and Engineering

Citation (APA)
Yoon, J., Lee, S. H., Tieves, F., Rauch, M., Hollmann, F., & Park, C. B. (2019). Light-Harvesting Dye-Alginate Hydrogel for Solar-Driven, Sustainable Biocatalysis of Asymmetric Hydrogenation. *ACS Sustainable Chemistry and Engineering*, *7*(6), 5632-5637. https://doi.org/10.1021/acssuschemeng.9b01075

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ACS Sustainable Chemistry & Engineering

Letter

Cite This: ACS Sustainable Chem. Eng. 2019, 7, 5632−5637

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Light-Harvesting Dye−Alginate Hydrogel for Solar-Driven, Sustainable Biocatalysis of Asymmetric Hydrogenation

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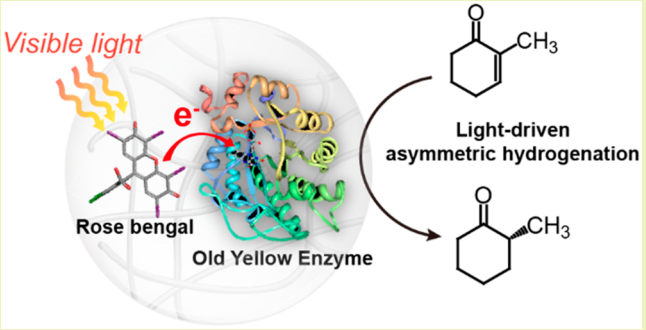
Supporting Information

ABSTRACT: We report visible light-driven, asymmetric hydrogenation of C═C bonds using an ene-reductase from *Thermus scotoductus* SA-01 (*Ts*OYE) and a light-harvesting dye (rose bengal, RB) co-immobilized in an alginate hydrogel. Highly efficient encapsulation of RB in alginate hydrogel was achieved using the intrinsic affinity between *Ts*OYE and RB, which allowed for the construction of robust RB-*Ts*OYE-loaded alginate capsules. In the absence of NADH, the photobiocatalytic system facilitated asymmetric reduction of 2-methylcyclohexenone to an enantiopure (*R*)-2-methylcyclohexanone (*ee* > 99%; max. conversion, 70.4%; turnover frequency, 1.54 min^{-1}; turnover number, 300.2) under illumination. A series of stability tests revealed a significant enhancement of *Ts*OYE's robustness in alginate hydrogel against heat and chemical denaturants. This study provides insight into a greener and sustainable approach of cofactor-free OYE catalysis for producing value-added chemicals using light energy.



KEYWORDS: *Photobiocatalysis, Ene-reductase, Asymmetric hydrogenation, NADH-free bioprocess, Alginate hydrogel*

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INTRODUCTION

Biocatalytic transformation is an attractive route for ecofriendly synthesis of chemicals. The outstanding catalytic activities and specificities (e.g., chemo-, regio-, stereo-, and enantioselectivities) of enzymes make them a green alternative to conventional catalysts for producing value-added chemicals under mild reaction conditions without generating unwanted byproducts.[1−3] Despite the fascinating features of biocatalysis, many challenges—such as limited stability of enzymes against environmental stresses, complex purification steps, and difficulties in reuse—remain in cost-effective and practical applications. The immobilization of biocatalysts is often considered as a solution to address the issues by enhancing enzyme stability and sustainability, allowing for easy recovery and reuse of key reaction components.[4−7]

Recent efforts of combining biocatalysis with photocatalysis take a step forward to greener and more sustainable synthesis of chemicals using solar energy.[8−12] In this study, we report an all-in-one photobiocatalytic platform for asymmetric hydrogenation driven by an ene-reductase from *Thermus scotoductus* SA-01 (*Ts*OYE) encapsulated in light-harvesting alginate hydrogel. The ene-reductases from the family of Old Yellow Enzymes (OYEs) catalyze chiral reduction of activated C═C double bonds.[13−15] During the biotransformation, the reduced form of OYE-bound flavin mononucleotide ($FMNH_2$) transfers two electrons and two protons to the activated C═C double bond via a Michael-type reaction, which is usually mediated by a reduced form of expensive nicotinamide adenine dinucleotide cofactor (NADH).[16,17] Different approaches for light-driven OYE-catalysis have been reported for the reduction of alkenes. For example, Burai et al. reported OYE-catalyzed reduction of ketoisophorone at a rate of approximately 20 $\mu M\ h^{-1}$ using quantum dots.[18] Recently, carbon nanodots were applied to OYE photobiocatalysis to enhance the reaction rate as high as 4 $mM\ h^{-1}$.[19] On the other hand, OYE-containing recombinant cyanobacteria were employed for light-driven alkene reduction with approximately 2 $mM\ h^{-1}$ of the production rate.[20]

Our previous work demonstrated that direct photoactivation of OYEs using xanthene derivatives, such as rose bengal (RB), as a photosensitizer could drive OYE-catalyzed reactions in a cofactor-free manner.[21] The simultaneous reduction of RB and *Ts*OYE facilitated the asymmetric reduction of C═C bonds in the absence of NADH, allowing for the synthesis of enantiopure (*ee* > 99%) products at the rate of 2.3 $mM\ h^{-1}$. Spectroscopic and electrochemical assays verified spontaneous binding of RB molecules—in a higher affinity than NADH—to

Received: February 22, 2019
Published: March 3, 2019

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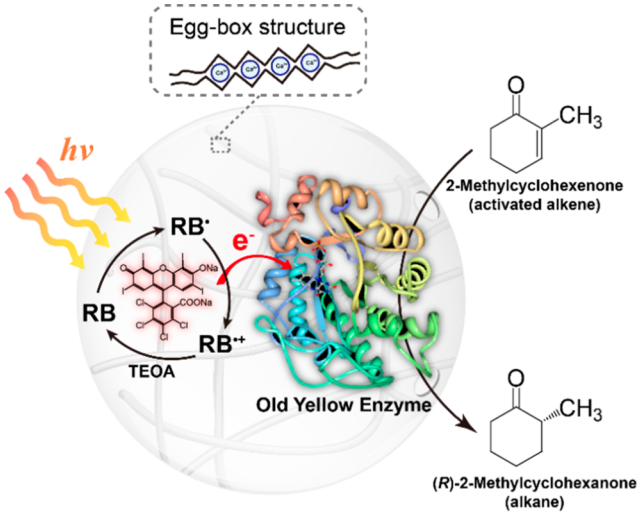
DOI: 10.1021/acssuschemeng.9b01075
ACS Sustainable Chem. Eng. 2019, 7, 5632−5637

the flavin prosthetic group of *Ts*OYE. In addition, the isoelectric point (pI) of *Ts*OYE (7.83) and pK_a values of RB (1.89 and 3.93) further indicate electrostatic affinity between *Ts*OYE and RB at pH 7.5.[22,23]

Alginate is a sea-weed-derived polysaccharide consisting of 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid blocks, which has been widely used for the encapsulation of biologicals because of low cost, biocompatibility, and facile gelation process. The ionic cross-linking, a most well-known method for forming alginate hydrogel, occurs through electrostatic interactions of the carboxyl groups of G blocks with divalent cations (e.g., Ca^{2+}, Ba^{2+}, Sr^{2+}), inducing an egg-box gel structure.[24] As depicted in Scheme 1, we encapsulated

Scheme 1. Schematic Illustration of a RB and *Ts*OYE Encapsulated Alginate Capsule for OYE Photobiocatalysis[a]



[a]The *Ts*OYE catalyzes asymmetric reduction of activated 2-methylcyclohexenone into (*R*)-2-methylcyclohexanone through photosensitization of RB in the alginate hydrogel.

*Ts*OYEs in the ionically cross-linked alginate beads, followed by a spontaneous post-hybridization of RB to the *Ts*OYEs in the hydrogel. The direct transfer of photoinduced electrons from RB to the enzyme-bound flavin prosthetic group using triethanolamine (TEOA) as an electron donor drives asymmetric reduction reactions, which occur within the alginate beads under illumination of a white light-emitting diode (LED).

RESULTS AND DISCUSSION

We simply treated the cell-free extract by a one-step heat treatment to obtain *Ts*OYEs for this study. *Ts*OYE-encapsulated alginate (*Ts*OYE/alginate) beads were prepared by mixing *Ts*OYEs with an aqueous alginate solution (2 w/v %), which was applied dropwise to a 100 mM $CaCl_2$ gelling medium (Figures S1, S2). The *Ts*OYE-containing beads exhibited a pale yellow color, distinguishable from pure alginate beads. According to our spectroscopic analyses (Figure S3), over 90% of encapsulated *Ts*OYE remained in alginate beads after 24 h of immersion in deionized water. The results show that alginate hydrogel encapsulates *Ts*OYEs stably within the beads without any significant leakage problem. We compared the loading efficiencies of RB in bare alginate and *Ts*OYE-containing alginate beads. For the experiments, we incubated the alginate beads in RB solutions for 1 h under mild stirring, collected supernatants, and measured the loading efficiency of RB using a spectrophotometer.

As shown in Figure 1A, the loaded amount of RB was different by a factor of 2 by the presence of *Ts*OYEs in the

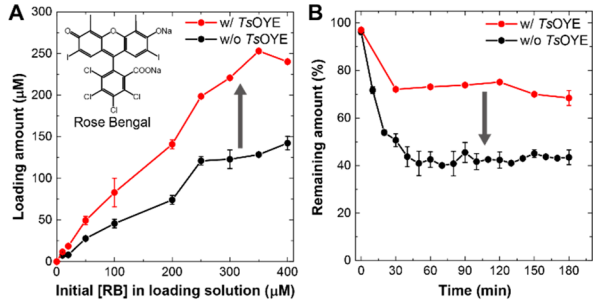


Figure 1. (A) RB-loading capacity of pure alginate beads and *Ts*OYE-containing alginate beads. (B) Remaining amount of RB in RB/alginate beads and RB-*Ts*OYE/alginate beads after 3 h of leaching test.

alginate hydrogel. We attribute the result to spontaneous association of RB molecules with *Ts*OYE in addition to physical entrapment of RB within the hydrogel. We also examined the effect of *Ts*OYE on the leaching of encapsulated RB by immersing RB/alginate and RB-*Ts*OYE/alginate beads in deionized water. More than a half of the RB molecules were lost after 3 h when loaded in bare alginate beads; in contrast, over 70% of loaded RB were retained in the *Ts*OYE/alginate hydrogel (Figure 1B). Previous studies reported the problem of encapsulating small molecules within the ionically cross-linked alginate hydrogels that have a relatively large pore size (~5 nm).[25,26] Thus, additional coatings (or hybridization) with other supporting materials have been pursued to enhance the efficacy of small molecule entrapment.[27−29] Our results indicate that facile immobilization of small RB molecules in alginate hydrogel can be achieved without any additional process using the intrinsic binding affinity between RB and *Ts*OYE. The stably encapsulated *Ts*OYE attracted RB molecules, resulting in a remarkably enhanced loading efficiency with a minimal leaching.

We investigated electrochemical responses of *Ts*OYE and RB encapsulated in alginate hydrogel using protein film voltammetry. We prepared a *Ts*OYE/alginate film on a glassy carbon electrode by a drop-casting method. As displayed in Figure 2A, a clear peak for the reduction of the prosthetic FMN group in *Ts*OYE was observed at −0.45 V (vs Ag/AgCl) in agreement with the literature.[30,31] Approximately 50 mV of a negative shift occurred in the presence of RB within alginate hydrogel, which indicates the interaction between RB and the immobilized *Ts*OYE. Additional analyses verified that alginate itself did not affect the electrochemical properties of RB and *Ts*OYE (Figure S4). The encapsulated *Ts*OYE and RB showed a distinguishable light response from that of either *Ts*OYE or RB (Figure 2B). At an applied potential of −0.6 V (vs Ag/AgCl), an anodic photocurrent of 0.6 μA was generated in the presence of both *Ts*OYE and RB in the alginate film. We attribute the result to the transfer of photoexcited electrons from RB to the enzyme-bound FMN, resulting in an increased anodic photocurrent. Note that the enzyme or RB alone did not show significant anodic photocurrents (<0.1 μA). The series of electrochemical and photochemical analyses verified

DOI: 10.1021/acssuschemeng.9b01075
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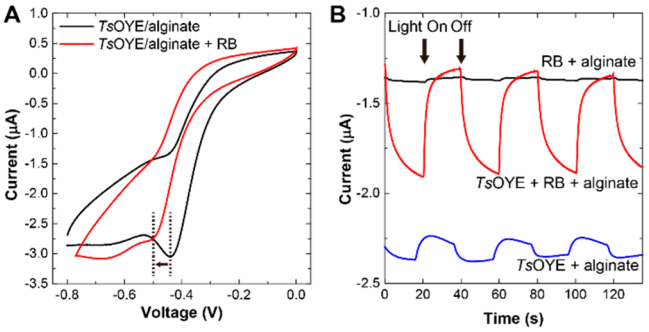

Figure 2. (A) Cyclic voltammetric diagrams of *Ts*OYE in alginate film in the presence and the absence of RB. (B) Photocurrent response of *Ts*OYE in alginate film at an applied potential of −0.6 V (vs Ag/AgCl, 3 M NaCl). A MOPS buffer (50 mM, pH 7.5) was used with Ag/AgCl and Pt as a reference electrode and a counter electrode, respectively.

the photosensitized, coreduction property of *Ts*OYE and RB in alginate hydrogel, which occurred through direct electron transfer between spontaneously hybridized *Ts*OYE and RB.

We studied catalytic activities of RB/*Ts*OYE hydrogels under illumination of a white LED (power: 5.22 mW/cm^2) using 2-methylcyclohexen-1-one (MCH) as a model substrate (10 mM). The RB-*Ts*OYE/alginate beads converted MCH into enantiopure (*ee* > 99%) (*R*)-2-methylcyclohexanone with 51% of conversion {[product]/[substrate]$_{initial}$ × 100 (%)} after 3 h of illumination (Figure 3A). We attribute the

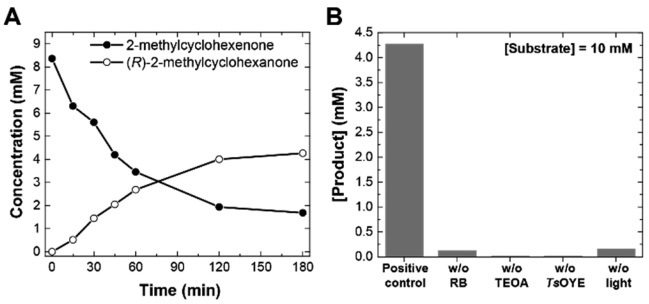

Figure 3. (A) Conversion of 2-methylcyclohexenone to (*R*)-2-methylcyclohexanone by RB-*Ts*OYE/alginate beads. Reaction conditions: [2-methylcyclohexenone] = 10 mM, [*Ts*OYE]$_{in\ alginate}$ = 21 μM, [RB]$_{in\ alginate}$ = 140 μM, 200 mM TEOA buffer (pH 7.5), reaction temperature = 30 °C, white LED illumination. (B) Control experiments about the effect of each reaction component. A 50 mM MOPS buffer (pH 7.5) was used for the without-TEOA case.

mismatch of mass balance between the substrate consumption and product formation to strong volatility of the chemicals at the reaction conditions. We also conducted a series of control experiments to test the role of key photobiocatalytic components [i.e., enzyme (*Ts*OYE), photosensitizer (RB), electron donor (TEOA), and light source].

As displayed in Figure 3B, a positive control (i.e., with all the components) generated 4.26 mM of enantiopure (*R*)-2-methylcyclohexanone, whereas none or a negligible amount (<0.15 mM) of the product was detected in the absence of any single component. The results show that RB-*Ts*OYE/alginate hydrogel functions as an efficient light-driven biocatalytic platform for asymmetric hydrogenation of C═C bonds. We also examined the dependency of product formation on TEOA concentration. As shown in Figure S5, the overall reaction rate was proportional to the concentration of TEOA up to 100 mM, which indicates that an excess amount of TEOA is necessary for the system. According to the literature,[19,32−34] a high surplus of electron donors (e.g., TEOA) is often needed to drive multiple electron-transfer steps in photocatalytic systems due to back electron transfer and charge recombination problems.

We further examined the effect of the loaded RB's concentration on the activities of *Ts*OYE. With the increasing amount of the loaded RB at a fixed *Ts*OYE concentration (21 μM) in alginate, the reaction conversion, turnover frequency (TOF), and turnover number (TON) increased gradually (Figure 4), which saturated to maximum values (66.9%

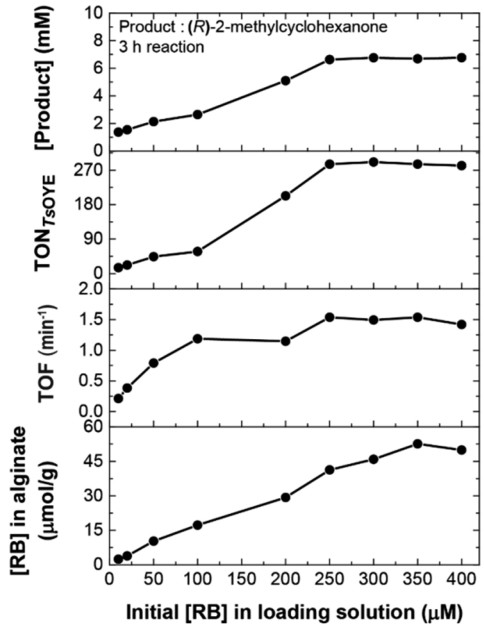

Figure 4. Photobiocatalytic activities of RB-*Ts*OYE/alginate beads with different initial RB concentrations in the loading solution. TOF and TON$_{TsOYE}$ values for the reduction of 2-methylcyclohexenone were determined after 30 min and 3 h of reactions, respectively.

conversion, 1.54 min^{-1} TOF, and 291.2 TON) due to the limitation of the RB-loading capacity (~51 μmol/g) within alginate beads. Free *Ts*OYE and RB dissolved in a buffer solution showed 78.8% conversion at the saturation point (RB/*Ts*OYE = 5) (Figure S6), similarly to our previous report (i.e., 76% conversion at RB/*Ts*OYE = 5).[21] In contrast, a lower conversion (66.9%) and a higher RB/*Ts*OYE molar ratio (RB/*Ts*OYE = 9.5) were observed at the saturation point of the initial reaction rate for the *Ts*OYE encapsulated in the alginate hydrogel. According to the literature,[4] encapsulation within a hydrogel matrix limits the enzyme's mobility, blocks the active site of the enzyme, and hinders mass transfer of substrates and products. We attribute the almost 2-fold increase in the saturation RB/*Ts*OYE ratio to the suppressed *Ts*OYE mobility and substrate accessibility that should cause an increase in the required RB amount in the hydrogel to reach the saturation point.

The *Ts*OYEs encapsulated in alginate hydrogel exhibited significantly enhanced stability against external stresses (e.g., heat, chemical denaturants). When we conducted stability tests in terms of relative conversion (RC = conversion/conversion$_{max}$ × 100), the immobilized *Ts*OYE showed strong

DOI: 10.1021/acssuschemeng.9b01075
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robustness against heat (RC ~ 80% at 60 °C) (Figure 5A), whereas free *Ts*OYE lost over a half of its initial conversion

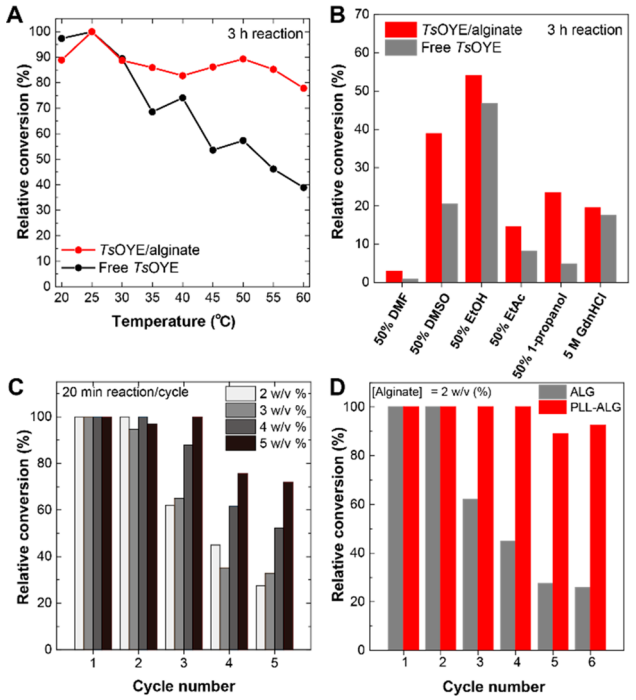

Figure 5. Series of stability tests for RB-*Ts*OYE/alginate beads in terms of relative conversion upon (A) temperature change and (B) solvent addition. Series of recycle tests for RB-*Ts*OYE/alginate beads by (C) varying alginate concentration and (D) coating a poly-L-lysine layer. [Substrate] = 10 mM, [TEOA] = 200 mM, reaction temperature = 30 °C.

(RC ~ 40% at 60 °C). Note that the loss of free *Ts*OYE's catalytic activity during prolonged incubation at high temperature has been reported previously. According to the literature,[35] residual activity of *Ts*OYE decreased gradually with the increasing temperature due to an unknown reason. The volatility issue of the substrate and product further contributes to the low conversion of *Ts*OYE-driven catalysis at high temperature. Furthermore, the immobilization also enhanced *Ts*OYE's resistance to chemical denaturants such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol (EtOH), ethyl acetate (EtAc), 1-propanol, and guanidine hydrochloride (GdnHCl) (Figure 5B). In particular, we observed significant enhancement of *Ts*OYE stability against DMSO and 1-propanol with doubled and quadrupled RC values, respectively. Our results show that *Ts*OYE becomes robust against heat and chemical denaturants when encapsulated in alginate beads. We further investigated the reusability of RB-*Ts*OYE/alginate beads through a series of recycling experiments. The RB-*Ts*OYE/alginate beads (alginate concentration, 2 w/v%) showed a good retention of RC in the first few cycles but lost most of their initial conversion over nine reaction cycles (with only about 10% of remaining RC) (Figure S7). We ascribe the result to the degradation of RB through irreversible photobleaching under illumination.[36−38] Figure S8 supports RB's photobleaching problem accelerated in the presence of TEOA, the electron donor.

Furthermore, a partial leakage of RB shown in Figure 1B became more significant with the increasing number of cycles. We attempted to enhance the reusability by (1) increasing alginate concentration, (2) supplementing fresh RBs, and (3) coating alginate beads with a poly-L-lysine layer.

As shown in Figure 5C, higher alginate concentration reduced RB leakage significantly by forming a denser gel network and improved the recyclability of RB-*Ts*OYE/alginate beads with a retention of RC over 50% after five cycles. We also found a dramatic enhancement of reusability—nearly 100% of its initial conversion until the ninth cycle—by supplementing fresh RBs (Figure S7). In addition, the electrostatic interaction between poly-L-lysine and RB at pH 7.5 could reduce the loss of RB, resulting in approximately 90% RC after six cycles (Figure 5D). To sum up, the long-term reusability of RB-*Ts*OYE alginate hydrogel was hampered by the intrinsic limitation of RB. Future studies will focus on addressing the photoresistance issue and the scalability of the light-driven biocatalytic process to a preparative level. For example, carbon nanotubes[39] and carbon-based quantum dots[34,40] are promising alternatives due to their resistance to photobleaching. Also, phosphine oxide-containing fluorescein, which is derived from xanthene dyes similar to RB, exhibited a superior photobleaching resistance according to the literature.[41] Finally, chemically modified RB derivatives with hopefully increased photobleaching stability may also represent an attractive solution.

We further tested a cell-free extract instead of heat-purified *Ts*OYE to demonstrate the sustainability of the system. We found that the cell-free extract shows a similar negative shift in the presence of RB, which indicates an electrochemical interaction between the cell-free extract and RB within an alginate hydrogel (Figure S10A, B). We also observed visible light-driven, cell-free-extract-catalyzed reduction of 2-methylcyclohexenone into an enantiopure (*ee* > 99%) product, though the overall catalytic activity was lowered (17.4% conversion and 53.5 TON_{TsOYE}) than that of heat-purified *Ts*OYE (Figure S10C, D). Overall, our results show that the RB-*Ts*OYE/alginate system is applicable to a cell-free extract and support the sustainability of the photobiocatalytic platform.

CONCLUSION

NADH-free, light-driven *Ts*OYE biocatalysis for asymmetric hydrogenation is demonstrated using cost-effective and biocompatible alginate hydrogel. The spontaneous RB-*Ts*OYE association within alginate hydrogel matrix enabled efficient and stable immobilization of both RB and *Ts*OYE, resulting in the formation of light-harvesting all-in-one beads with excellent catalytic performances of *Ts*OYE. The reduction of 2-methylcyclohexenone was achieved through direct electron transfer and coreduction of *Ts*OYE and RB in alginate hydrogel. The RB-*Ts*OYE/alginate beads exhibited a catalytic performance of maximum 70.4% conversion of (*R*)-2-methylcyclohexanone (*ee* > 99%) and 300.2 TON_{TsOYE}, while the limited mobility of *Ts*OYE and substrate accessibility induced a 2-fold increase in the saturation ratio of RB/*Ts*OYE compared to the free enzyme. We revealed enhancements of stability and reusability of immobilized RB and *Ts*OYE, which hints at greener applications of OYEs for solar-driven synthesis of value-added chemicals.

DOI: 10.1021/acssuschemeng.9b01075
ACS Sustainable Chem. Eng. 2019, 7, 5632−5637

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b01075.

Experimental details for *Ts*OYE preparation and encapsulation in alginate hydrogel, optical images of alginate beads, spectroscopic analyses for *Ts*OYE leakage test, additional cyclic voltammograms of alginate film, time course data and control experiments for OYE photobiocatalysis, and examination of RB's photobleaching. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Research Foundation (NRF) via the Creative Research Initiative Center (Grant NRF-2015 R1A3A2066191), Republic of Korea, for C.B.P. and The Netherlands Organisation for Scientific Research by a VICI grant (Grant 724.014.003) for F.H.

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