Effect of Lipase Ionic Liquid Coating and Imprinting on Transesterification and Thermostability

M. Matsumoto* **and Y. Tahara**
Department of Chemical Engineering
and Materials Science, Doshisha University,
Kyotanabe, Kyoto 610-0321, Japan

doi: https://doi.org/10.15255/CABEQ.2024.2334
Original scientific paper
Received: May 10, 2024
Accepted: September 22, 2024

This study investigates the effects of the treatments (substrate analogue imprinting and ionic liquid coating) on the catalytic activity and thermostability of lipase from *Burkholderia cepacia* in the transesterification of vinyl acetate with benzyl alcohol. The catalytic activity was significantly enhanced when the lipase was coated with a Brij 56-based ionic liquid or surfactant Brij 56. Notably, the ionic liquid coating had a greater effect than the imprinting treatment. The activity of the ionic liquid-coated lipase was over 50 times greater than that of the free lipase. Additionally, after incubation at 90 °C for 2 hours, the residual activity of the ionic liquid-coated lipase was about twice those of the other lipases. These findings demonstrate that the ionic liquid coating substantially improved both the reactivity and thermostability of the lipase compared to the native lipase.

Key words
lipase, ionic liquid-coating, imprinting, thermostability

Introduction

Lipases are widely distributed in animals, plants, and microorganisms. However, current applied research predominantly focuses on microbial lipases, except in the fields of medicine and pharmacology. This is due in part to microbial lipases' ability to stably express their functions without the need for cofactors, as well as their ease of purification. The industrial production of microbial lipases has been explored across various fields, leading to the development of efficient production technologies and the purification of numerous types of microbial lipases. Comparative studies have demonstrated the diversity of lipases due to differences in their sources, potentially expanding the range of lipase applications[1]. Among the lipases from various sources, the lipase from *Burkholderia cepacia* is widely recognized and commercially available, known for its thermal resistance and tolerance to a wide range of solvents and short-chain alcohols[2]. Its primary application is in transesterification reactions in hydrophobic organic solvents [2].

One method for the activation of enzymes in organic solvents is imprinting, which involves lyophilizing lipase loaded with a substrate analogue to form a lipase-substrate analogue complex, followed by the removal of the substrate analogue[3]. This process helps maintain the lipase in an active conformation. In aqueous solutions, the active sites of lipases are covered by a flexible region or "lid". Interfacial activation by organic solvents or amphiphiles can induce the lid to open, making the active site accessible[4]. Imprinted lipases typically exhibit much higher activity than their unimprinted counterparts.

Previous studies have investigated the effects of imprinting on the reactivity and thermostability of lipases from *Candida rugosa*[5–7] and porcine pancreas[8] in the esterification and transesterification reactions in organic solvents. The effect of imprinting on esterification has also been reported for immobilized *Burkholderia cepacia* lipases[9,10]. For instance, the relative activity of imprinted lipase incubated at 60 °C showed no decline after an initial period, indicating a significant improvement in its thermostability. In contrast, the relative activity of free lipase decreased to 0.53 after 4 hours[9]. However, these studies on the thermostability of imprinted lipases were qualitative rather than quantitative.

Another method for activating enzyme function in organic solvents proposed recently involves coating the enzyme with an ionic liquid. Several studies have reported on sucessful ionic liquid-coating of lipases from *Burkholderia cepacia*. Lee *et al*. reported a significant increase in enantioselectivity and activity in the transesterification reaction by lipase coated with their prepared ionic liquids[11–13]. Itoh *et al.* systematically studied various ionic liquids suitable for lipase coating and explored their properties in transesterification reactions using ionic liquid-coated lipases[14–18]. One of the ionic liq-

*Corresponding author; Tel/Fax +81-774-65-6655;
E-mail: mmatsumo@mail.doshisha.ac.jp

uid-coated lipases they prepared is now commercially available. Furthermore, ester synthesis[19] and ring-opening polymerization[20] by ionic liquid-coated lipases from *Candida antarctica* have also been reported. However, these studies focused primarily on synthetic organic chemistry and lacked quantitative treatment.

In this study, we quantitatively investigated the effects of substrate analogue imprinting and ionic liquid coating on the catalytic activity and thermostability of *Burkholderia cepacia* lipase in the transesterification of vinyl acetate with benzyl alcohol.

Experimental methods

Materials

Lipase from *Burkholderia cepacia* (Amano Lipase PS, 534641, ≥30,000 U g^{-1}, Sigma-Aldrich) and an ionic liquid (1-butyl-2,3-dimethylimidazolium poly [oxyethylene (10)] cetyl sulfate) – coated lipase (B3028, Tokyo Chem. Ind. Co.) prepared from Brij 56 and Amano Lipase PS were used throughout the experiments. A lipase coated with surfactant Brij56 (polyoxyethylene (10) cetyl ether, Sigma-Aldrich) was also employed for comparison. Vinyl acetate and benzyl alcohol served as substrates in the transesterification reactions. The solvents used for the transesterification included benzene, toluene, ethylbenzene, cyclohexane, hexane, diphenyl ether, and isooctane were. These solvents are commonly used in organic solvents-based transesterifications. Hexane, used in the imprinting process, was dried with 3 Å molecular sieves prior to use. All reagents were of analytical grade and used without further purification.

Imprinting of lipases

The imprinting of the native lipase and the ionic liquid-coated lipase with propionic acid as the imprint molecule allowed the method previously described in references[5–8]. Lipase (500 mg) was dissolved in 9 cm^3 of a phosphate buffer (pH 7). A solution of propionic acid (3.5 mmol) and Tween 80 (1000 mg) was prepared in 10 cm^3 of ethanol. To the lipase solution, 1 cm^3 of acid solution was added, and the mixture was incubated for 20 min at room temperature. The solution was then freeze-dried, and the resulting powder was washed with hexane to remove the acid. The resultant lipases were filtered and dried in vacuo. The same imprinting procedure was applied to the ionic liquid-coated lipase.

Ionic liquid-coated lipase

A commercial ionic liquid-coated lipase was used for the experiment. For comparison, a Brij 56-coated lipase was prepared following a similar procedure to that used in a previous report on ionic liquid-coated lipases[15]. Fig. 1 summarizes the various lipase pretreatments used in this study.

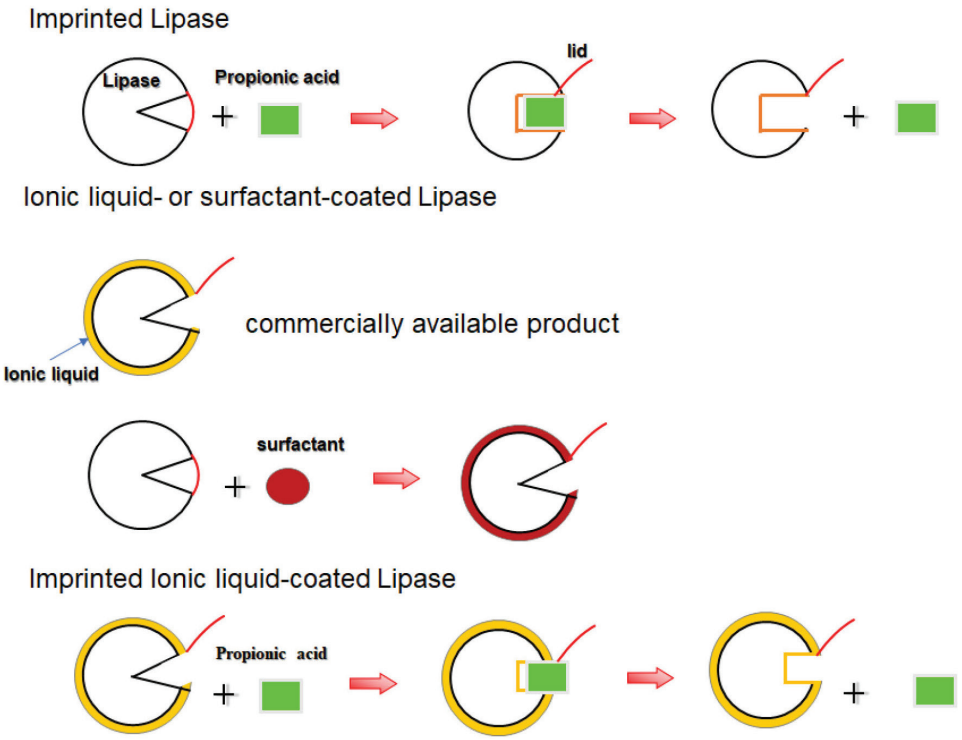

Fig. 1 – *Schematic diagram of lipase pretreatment process*

Transesterification

The transesterification reaction with vinyl acetate and benzyl alcohol was used as a model reaction to assess the effects of ionic liquid coating and imprinting. The reactions were initiated by adding 1.0 mg of lipase powder to 1 mL of organic solvent containing both substrates (vinyl acetate and benzyl alcohol) in vials at 50 °C, and stirring at 1000 rpm. At regular intervals over 120 minutes, the vials were removed from the thermomixer (Comport, Eppendorf), immediately centrifuged, and the extent of the reaction was determined by measuring the benzyl acetate concentration using HPLC. The initial experimental conditions were set at a vinyl acetate concentration of [VA] = 300 mmol dm^{-3} and benzyl alcohol concentration of [BA] = 300 mmol dm^{-3}. Reactivity was defined as a relative value based on the conversion rate of free lipase after 30 minutes.

Thermostability of lipase

The lipases were incubated in isooctane at 90 °C. After the prescribed incubation period, the substrate solution was added, and the reaction was carried out at 50 °C and 1000 rpm for 30 min. Samples were collected to measure lipase activity, and residual activities were defined as the ratio of the initial reaction rates of the incubated lipases to those without incubation.

Residual activity, a_t, is given by the following equation based on reversible first-order deactivation kinetics[27]:

$$\frac{a_t - a_\infty}{1 - a_\infty} = \exp\left\{-\left(k_d + k_r\right)t\right\} \qquad (1)$$

where k_d and k_r are lipase deactivation and refolding rate constants, respectively, a_∞ is the residual activity at equilibrium and t is the elapsed time.

Analysis

Standard solutions of benzyl acetate were used to prepare the calibration curves. The concentrations of benzyl acetate in the organic solutions were determined by HPLC (Shimadzu LC-10ADvp) with a Wakosil-II 5C18AR column and an eluent solution (acetonitrile:water = 1:1) as a mobile phase (0.6 cm^3 min^{-1}). Esters were identified using a UV detector set at 255 nm (Shimadzu SPD10AV).

Results and discussion

Effect of pretreatment on transesterification

The effect of various lipase pretreatments, such as imprinting of free lipase, ionic liquid-coating, and imprinting of ionic liquid-coated lipase in the transesterification reaction between vinyl acetate and benzyl alcohol was investigated. Fig. 2 shows a typical example of the conversion changes over time during the transesterification reaction using untreated (free) lipase and pretreated (imprinted, ionic liquid-coated, and imprinted ionic liquid-coated) lipases. The pretreatments resulted in a significant increase in conversion rate. After 2 hours, the conversions reached constant values regardless of the type of pretreatment applied. This indicated that the lid of the lipase was open in all the pretreatment cases, as shown in Fig. 1. Interestingly, imprinting of the ionic liquid-coated lipase had no positive effect on reaction activity. The effect of pretreatment on the relative reactivity at 30 min in hexane using free lipase is shown in Fig. 3. The activity of the ionic liquid-coated lipase was more than 50 times higher than that of the free lipase. Transesterification reactions of secondary alcohols with vinyl acetate in diisopropyl ether catalyzed by ionic liquid-coated lipase have been reported[15]. The ionic liquid-coated lipase showed a 500- to 1000-fold acceleration effect on a few secondary alcohols with naphthalene rings, while the acceleration for most other secondary alcohols ranged between 1- to 98-fold. In the previous report[15], there is no example using primary alcohols, but the degree of acceleration was 15-fold with 1-phenylethanol. It was found that even primary alcohols provide sufficient acceleration in transesterification reactions. The lipase coated with surfactant Brij-56, a component of ionic liquid, also showed high activity. The imprinted lipase significantly enhanced activity, although it was less effective than the coated lipase. Fig. 4 shows the relationships between the relative activities of the free and ionic liquid-coated lipases and

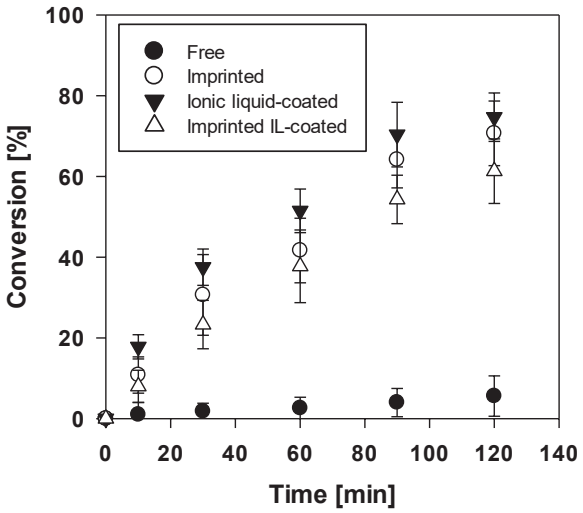

Fig. 2 – *Effect of lipase pretreatment (imprinted, ionic liquid-coated, and imprinted ionic liquid-coated) on conversion of transesterification in cyclohexane: Vinyl acetate concentration = benzyl alcohol concentration = 300 mmol dm^{-3}*

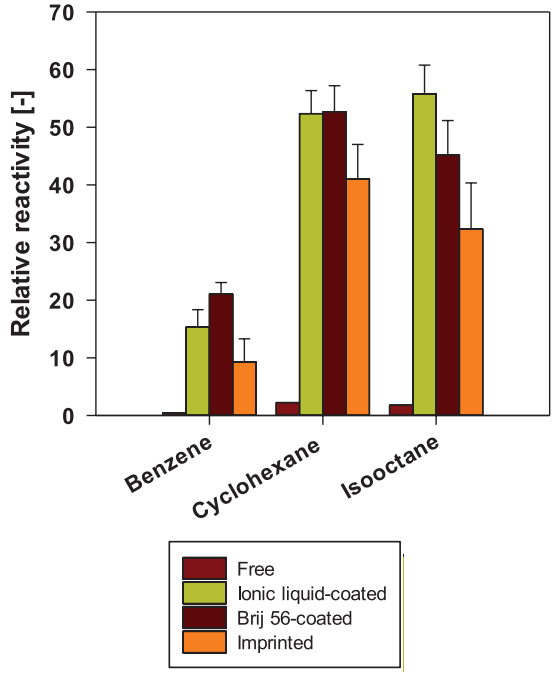

Fig. 3 – *Effect of lipase pretreatment (imprinted, ionic liquid-coated, and Brij 56 coated) and solvent on relative activity of transesterification. Vinyl acetate concentration = benzyl alcohol concentration = 300 mmol dm^{-3}.*

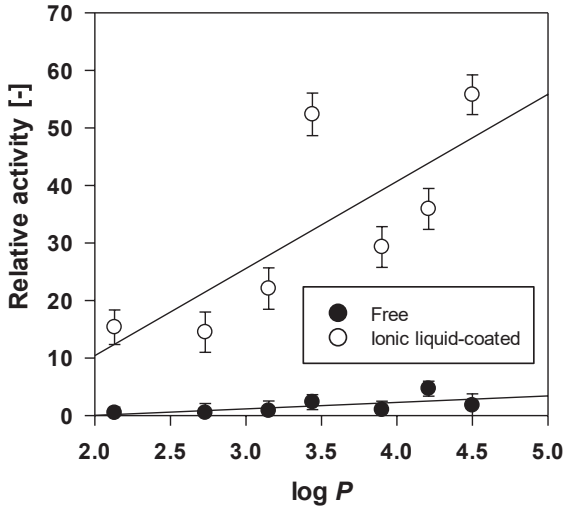

Fig. 4 – *Relationship between transesterification activity of free and ionic liquid-coated lipases and solvent hydrophobicity. Vinyl acetate concentration = benzyl alcohol concentration = 300 mmol dm^{-3}.*

the hydrophobicity of the solvents (log *P*). The relative activity increased roughly linearly with increasing log *P* values, which differs from previous report using this lipase where the transesterification reaction between the vinyl acetate and the 2-*o*-benzylglycerol preferred solvents with lower log *P* values[21], and that the esterification reaction between lauric acid and dodecanol was not significantly dependent on the log *P* of the solvent[22]. Generally, the catalytic activity of lipase increases as the substrate (log *P* = 1.1 for benzyl alcohol, 0.3 for 2-*o*-benzylglycerol) and the solvent hydrophobicities decrease because of the preferential-partitioning behavior of the substrate between the reaction medium and the active sites of the lipase. Esterification and transesterification catalyzed by lipases are known to follow the Ping-Pong mechanism[23]. The lipase picks up an acyl-group from the vinyl acetate, forms a lipase-acyl complex and releases vinyl alcohol. This acyl-enzyme then reacts with benzyl alcohol to form benzyl acetate, and the lipase returns to its original state. Previous studies have focused on the relationship between the reactivity and the hydrophobicity of substrates with acyl groups, in which case the alcohol substrate was not hydrophobic. Therefore, when a hydrophobic alcohol component is used as in this study, a more hydrophobic environment is considered suitable for the reaction between the acyl enzyme and the alcohol.

Effect of lipase pretreatment on its thermostability

Fig. 5 shows the time course of residual activities of lipases in cyclohexane at 90 °C. Due to their excellent heat resistance[2], neither free nor pretreated lipase showed deactivation within 2 hours with in the temperature range of 50 – 70 °C. Therefore, for this study, the incubation temperature was set at a high level of 90 °C. In all lipase formulations, enzyme activity gradually decreased at 120 min and then remained constant, suggesting that enzyme deactivation followed first-order reversible kinetics.

When the hydrolysis reaction in water is used as an indicator of lipase activity, the deactivation of lipase from *Burkholderia cepacia* follows irreversible first-order kinetics[24]. Moreover, when the transesterification reaction in dichloromethane is used to

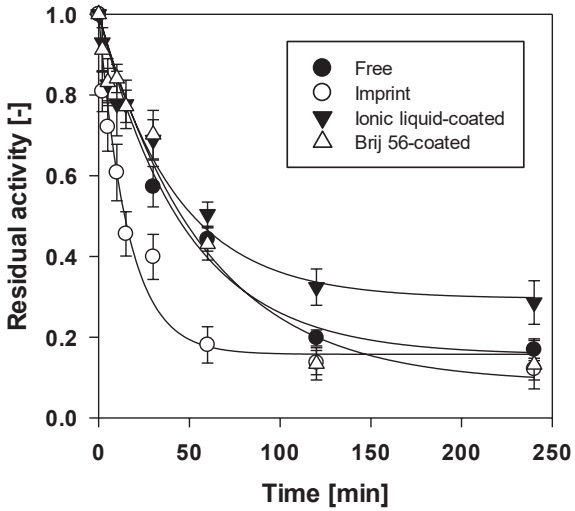

Fig. 5 – *Thermostability of pretreated lipases (imprinted, ionic liquid-coated, and Brij 56 coated) at 90 °C in isooctane. Solid lines were calculated using Eq. (1).*

Table 1 – *Parameters in the lipase deactivation kinetics based on Equation (1) at 90 °C in isooctane*

90 °C	Native	Imprinted	IL-coated	Brij 56-coated
a_∞	0.156±0.034	0.159±0.037	0.296±0.045	0.0861±0.0589
$10^2\ k_d$ (min^{-1})	1.76±0.17	5.29±0.65	1.67±0.23	1.58±0.19
$10^2\ k_r$ (min^{-1})	0.326±0.105	0.989±0.336	0.702±0.213	0.149±0.022

evaluate the activity of the immobilized lipase from *Burkholderia cepacia*, its authors recommend an irreversible series model for the deactivation of this lipase, which is not very different from the results obtained with irreversible first-order kinetics[25]. This may arise because lipase exposure to the nonpolar organic phase prevents complete deactivation due to a more stable, open form of the catalytically active site than in water[26].

The parameters, k_d, k_r and a_∞ included in Eq. (1) were calculated for the data in Fig. 5 using Sigma Plot 14.0, with the results shown in Table 1. The calculated lines (solid) are in good agreement with the experimental results, confirming the validity of the model in Eq. (1). Fig. 5 shows that the thermostability of the imprinted lipase decreased, which corresponds to a three-fold increase in the deactivation constant k_d compared to the other lipases in Table 1.

Although the inactivation kinetics have not been analyzed, the thermostability of the immobilized lipase from *Burkholderia cepacia* has been reported to increase by imprinting[9,10], whereas, in this study, the thermostability of the free lipase conversely decreased by imprinting. This difference may be attributed to the greater rigidity of the external backbone in immobilized lipase molecules[10].

This differs from the findings with porcine pancreas lipase[8], where the effect was attributed to the origin of the lipase. On the other hand, there was no significant difference in the deactivation constants of the free lipase, the Brij 56-coated lipase and the ionic liquid-coated lipase, as shown in Fig. 5 and Table 1. The residual activity of the ionic liquid-coated lipase after 2 hours was about twice as high as that of the other lipases. The fact that the ionic liquid-coated enzyme does not lead to complete inactivation may be a major advantage.

Conclusions

This study investigated the effects of imprinting and ionic liquid-coating on the catalytic activity and thermostability of *Burkholderia cepacia* lipase in transesterification reactions in organic solvents. Both imprinting and ionic liquid-coating significantly enhanced catalytic activity, although no synergistic effect between the ionic liquid coating and imprinting was observed. The effect of the former outstripped that of latter. The transesterification activity between vinyl acetate and benzyl alcohol increased with greater hydrophobicity of the solvent, contrary to previous reports. Furthermore, the thermostability of the lipases at 90 °C was greatly enhanced by coating the lipase with Brij 56 and ionic liquid. On the other hand, imprinting notably reduced the thermostability of the lipase, although the thermostability of the porcine pancreas lipase was enhanced by imprinting. The effect of imprinting was also found to be dependent on enzyme origin. As a result, the ionic liquid-coated enzyme showed improved transesterification activity and thermostability.

ACKNOWLEDGMENTS

We thank Ryosuke Yamada for his experimental assistance. This work was supported by a research grant from JSPS KAKENHI Grant Number 23K04480.

References

1. *Yamane, T.*, Reactions catalyzed by lipid-related enzymes, J. Japan. Oil Chem. Soc. **40** (1991) 965. doi: https://doi.org/10.5650/jos1956.40.965
2. *Sánchez, D. A., Tonetto, G. M., Ferreira, M. L.*, *Burkholderia cepacia* lipase: A versatile catalyst in synthesis reactions, Biotechnol. Bioeng. **115** (2018) 6. doi: https://doi.org/10.1002/bit.26458
3. *Gupta, M. N.*, Non-aqueous Enzymology: Issues and Perspectives, Gupta, M. N., ed., Methods in Non-aqueous Enzymology, 2000, Birkhäuser Verlag, Bazel (2000). doi: https://doi.org/10.1007/978-3-0348-8472-3
4. *González-Navarro, H., Braco, L.,* Improving lipase activity in solvent-free media by interfacial activation-based molecular bioimprinting, J. Mol. Catal. B **3** (1997) 111. doi: https://doi.org/10.1016/S1381-1177(96)00038-0
5. *Matsumoto, M., Matsui, E.,* Enhanced activities and thermostability of lipase pretreated with carboxylic and perfluorocarboxylic acids in transesterification, J. Chem. Tech. Biotechnol. **93** (2018) 3219. doi: https://doi.org/10.1002/jctb.5678
6. *Matsumoto, M., Hasegawa, Y.,* Enzyme kinetics of solvent-free esterification with bio-imprinted lipase, Chem. Biochem. Eng. Q. **33** (2019) 495. doi: https://doi.org/10.15255/CABEQ.2019.1692
7. *Matsumoto, M., Nakao, K., Tahara, Y.,* Effect of imprinting and water activity on transesterification and thermostability with lipases in ionic liquid, Chem. Biochem. Eng. Q. **35** (2021) 57. doi: https://doi.org/10.15255/CABEQ.2020.1899

8. *Matsumoto, M., Tahara, Y.,* Enhanced esterification activity and thermostability of imprinted(poly(ethylene glycol)-lipase complex, Chem. Biochem. Eng. Q. **37** (2023) 255. doi: https://doi.org/10.15255/CABEQ.2022.2205
9. *Cao, X., Yang, J., Shu, L., Yu, B., Yan, Y.*, Improving esterification activity of *Burkholderia cepacia* lipase encapsulated in silica by bioimprinting with substrate analogues, Process Biochem. **44** (2009) 177. doi: https://doi.org/10.1016 /j.procbio.2008.10.003
10. *Liu, T., Liu, Y., Wang, X., Li, Q., Wang, J., Yan, Y.*, Improving catalytic performance of *Burkholderia cepacia* lipase immobilized on microporous resin NKA, J. Mol. Catal. B-Enz. **71** (2011) 45. doi: https://doi.org/10.1016/j.molcatb.2011.03.007
11. *Lee, J. K., Kim, M. J.*, Ionic liquid-coated enzyme for biocatalysis in organic solvent, J. Org. Chem. **67** (2002) 6845. doi: https://doi.org/10.1021/jo026116q
12. *Lee, J. K., Kim, M. J.*, Room temperature solid-phase ionic liquid-immobilized enzyme for biocatalysis in organic solvent. Markedly enhanced enantioselectivity, Bull. Korean Chem. Soc. **32** (2011) 3149. doi: https://doi.org/10.5012/bkcs.2011.32.8.3149
13. *Lee, J. K.*, Immobilization of enzyme on hydrophobic room temperature solid-phase ionic liquids and its use for biocatalytic transesterification in organic solvent. Markedly enhanced activity of lipase, Bull. Korean Chem. Soc. **33** (2012) 3458. doi: https://doi.org/10.5012/bkcs.2012.33.10.3458
14. *Itoh, T., Han, S., Matsushita, Y., Hayase, S.,* Enhanced enantioselectivity and remarkable acceleration on the lipase-catalyzed transesterification using novel ionic liquids, Green Chem. **6** (2004) 437. doi: https://doi.org/10.1039/b405396f
15. *Itoh, T., Matsushita, Y., Abe, Y., Han, S., Wada, S., Hayase, S., Kawatsura, M., Takai, S., Morimoto, M., Hirose, Y.,* Increased enantioselectivity and remarkable acceleration of lipase-catalyzed transesterification by using an imidazolium PEG-alkyl sulfate ionic liquid, Chem. Eur. J. **12** (2006) 9228. doi: https://doi.org/10.1002/chem.200601043
16. *Abe, Y., Yoshiyama, K., Yagi, Y., Hayase, S., Kawatsura, M., Itoh, T.,* A rational design of phosphonium salt type ionic liquids for ionic liquid coated-lipase catalyzed reaction, Green Chem. **12** (2010) 1976. doi: https://doi.org/10.1039/c0gc00151a
17. *Abe, Y., Yagi, Y., Hayase, S., Kawatsura, M., Itoh, T.,* Ionic liquid engineering for lipase-mediated optical resolution of secondary alcohols: Design of ionic liquids applicable to ionic liquid coated-lipase catalyzed reaction, Ind. Eng. Chem. Res. **51** (2012) 9952. doi: https://doi.org/10.1021/ie202740u
18. *Matsubara, Y., Kadotani, S., Nishihara, T., Hikino, Y., Fukaya, Y., Nokami, T., Itoh, T.*, Phosphonium alkyl PEG sulfate ionic liquids as coating materials for activation of *Burkholderia cepacia* lipase, Biotechnol. J. **10** (2015) 1944. doi: https://doi.org/10.1002/biot.201500413
19. *Mutschler, J., Rausis, T., Bourgeois, J., Bastian, C., Zufferey, D., Mohrenz, I. V., Fisher, F.,* Ionic liquid-coated immobilized lipase for the synthesis of methylglucose fatty acid esters, Green Chem. **11** (2009) 1793. doi: https://doi.org/10.1039/b916016g
20. *Dong, F., Zhang, L., Tong, X., Chen, H., Wang, X., Wang, Y.*, Ionic liquid coated lipase: Green synthesis of high molecular weight poly(1,4-dioxan-2-one), J. Mol. Catal B-Enz. **77** (2012) 46. doi: https://doi.org/10.1016/j.molcatb.2012.01.006
21. *Hazarika, S., Goswami, P., Dutta, N. N.*, Lipase catalysed transesterification of 2-*o*-benzylglycerol with vinyl acetate: Solvent effect, Chem. Eng. J. **94** (2003) 1. doi: https://doi.org/10.1016/s1385-8947(02)00320-0
22. *Pan, S., Liu, X., Xie, Y., Yi, Y., Li, C., Yan, Y., Liu, Y.*, Esterification activity and conformational studies of *Burkholderia cepacia* lipase in conventional organic solvents, ionic liquids and their co-solvent mixture media, Bioresour. Technol. **101** (2010) 9822. doi: https://doi.org/10.1016/jbiortech.2010.07.107
23. *Bornadel, A., Åkerman, C. O., Adlercreutz, P., Hatti-Kaul, R., Borg, N.*, Kinetic modeling of lipase-catalyzed esterification reaction between oleic acid and trimethylolpropane: A simplified model for multi-substrate multi-product Ping-Pong mechanisms, Biotechnol. Prog. **29** (2013) 1422. doi: https://doi.org/10.1002/btpr.1806
24. *Ortega, N., Sáez, L., Palacios, D., Busto, M. D.*, Kinetic modeling, thermodynamic approach and molecular dynamics simulation of thermal inactivation of lipase from *Burkholderia cepacia* and *Rhizomucor miehei*, Int. J. Mol. Sci. **23** (2022) 6828. doi: https://doi.org/10.3390/ijms23126828
25. *Hazarika, S., Dutta, N. N.*, Transesterification of 2-*o*-benzylglycerol with vinyl acetate by immobilized lipase: Study of reaction and deactivation kinetics, Org. Process Res. Dev. **8** (2004) 229. doi: https://doi.org/10.1021/op030040y
26. *De Oliveira, I. P., Jara, G. E., Martínez, L.*, Molecular mechanism of activation of *Burkholderia cepacia* lipase at aqueous-organic interfaces, Phys. Chem. Chem. Phys. **19** (2017) 31499. doi: https://doi.org/10.1039/c7cp04466f
27. *Sanada, A.,* Enzyme deactivation, Biotechnol. Adv. **6** (1988) 349. doi: https://doi.org/10.1016/0734-9750(88)91890-3