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REVIEW

Water Activity Control in Lipase-catalyzed Reaction System

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Abstract This mini review describes the effects of water activity (a_w) on the kinetics, regio- and enantioselectivities of lipases, and various methods for measuring and controlling a_w in lipase catalyzed reaction in organic solvent.

Key words: Lipase, water activity, regioselectivity, enantioselectivity

Hydrolytic enzymes can be used for the synthetic reactions if the equilibrium position is moved to synthesis. This can be accomplished by carrying out the reaction in organic solvent [14, 60]. The synthetic yield can be enhanced by continuously removing the water produced during the reaction. Many researchers have proposed several methods for water removal, such as headspace evacuation [7, 44], pervaporation [41], use of molecular sieve [17], salt hydrate pairs [26, 39], saturated salt solution, adsorption [16], and sparging of dry inert gas through the reaction medium [35]. However, continuous water removal might result in the enzyme inactivation when the water content is too low. Therefore, a correct water content must be maintained when biocatalysts are used in organic solvents because the physical properties of the enzymes have been varied depending on the hydration state of the enzyme [24, 68]. In particular, synthetic activity of the lipase is greatly affected by the water content or water activity [22, 23]. Various changes of the reaction system alter the requirement for the optimal water content [27] because water in the reaction mixture interacts with different supports, organic solvents, and biocatalysts [45, 67, 68]. It is, therefore, advantageous to use the thermodynamic activity of water (a_w) instead of water content or water concentration to characterize the water effects in the reaction mixture. The water activity (a_w) is defined as follows:

$$a_w = f_w/f_w^o = x_w\gamma_w$$

where f_w and f_w^o are the fugacities of water in the mixture and in its standard state, respectively, and x_w and γ_w are the mole fraction and activity coefficient of water, respectively. The activity coefficient can be estimated using NRTL or UNIFAC equations [42]. If water activity is maintained at a constant level, there would be no change in the nature of the microenvironment of the lipases, and the profile of the lipase activity against a_w, therefore, would be unchanged. However, in the case of water content, the optimum would change depending on the amounts of biocatalyst, solvent, and substrate [26, 55, 57]. The influence of water activity on the reaction rate has been studied extensively [15, 29, 51, 56]. Lipases responded differently to water activity. Lipase from *Rhizopus arrhizus* showed optimal activity at low water activity (a_w=0.33). Lipase from *Pseudomonas* sp. shows the activity increase with increasing water activity, and other lipases have intermediate profiles with broader optima (Lipozyme and *Candida rugosa*) [61]. In the meantime, Wehtje and Adlercreutz [62] reported that the lipases showed similar water activity profiles in the different reaction systems (esterification, transesterification). This review summarizes the effects of the a_w on lipase catalysis and methods of a_w control.

Effects of Water Activity on Lipase Kinetics

The kinetics of the lipase is influenced by water activity because a_w is a key parameter which affects the enzyme activity. There are a few reports on the effects of water activity on apparent kinetic constants in lipase catalysis in organic media [9, 32, 54, 59]. Bovara *et al.* [9] published the V_{max} and K_m values of various forms of lipase from *Pseudomonas cepacia* (powder, immobilized by adsorption onto Celite or covalently linked to PEG) in the organic solvents at various water activities (less than 0.1 to 0.84). Lipase catalyzed transesterification of octanol with vinyl butyrate was performed as a model reaction. K_m for the nucleophile increased with

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increasing a_w for all three lipase forms. V_{max} increased with increasing a_w for PEG-lipase, whereas there was an optimum at intermediate a_w value (0.11~0.38) for lipase powder and Celite-immobilized lipase. Lately, lipase kinetics on water activity were studied in depth [61, 62]. They studied the catalytic activity of lipases (EC-3.1.1.3) from *Rhizopus arrhizus, Candida rugosa*, and *Pseudomonas* sp. in the organic media. Enzyme activity in organic media is normally measured by the initial reaction rate at a fixed substrate concentration. The results of the study indicated that hydrophobic solvents preserve enzyme activity longer than hydrophilic solvents, and that some lipases are more active at the lower water activity [55, 64]. On the contrary, measurement of V_{max} and K_m values showed that the lipases had a higher activity at higher a_w, although some, *i.e., R. arrhizus*, did have substantial activity at low a_w. Hydrophobic solvents gave low K_m and hydrophilic solvents high K_m, implying that the effect of the decreased activity at high a_w is probably due to variations in apparent K_m of the substrates [9, 61]. In the esterification reaction, the K_m for the alcohol increased when a_w was increased from 0.11 to 0.97 [61], implying that water acts as a competitive inhibitor to the alcohol. The V_{max} for the lipases increased with increasing a_w. When the reactions were performed at various substrate concentrations in the same order as the K_m values, the effect of high K_m value at high a_w was seen as a decline in the reaction rate, which created a bell-shaped activity profile. The conditions of the assay, *i.e.*, the substrate concentration, showed different profiles.

Effect of a_w on the thermostability of lipase was investigated by Turner *et al.* [52]. The thermostability of *Candida rugosa* lipase was higher in anhydrous environments compared to that of aqueous solution. An almost linear decrease in the denaturation temperature was obtained as increasing a_w was observed. They also showed that the stabilization of the lipase folding occurred with decreasing a_w.

Effects of a_w on Regioselectivity and Enantioselectivity

Lipases can be used for the production of regioselective and enantioselective pure compounds because of its specific nature [18]. Water activity affects the lipase activity, the substrate conversion, and the specific nature of lipase [3, 11, 30]. Lipase properties can be improved for the increases of the purity and product yield by controlling a_w. Fureby *et al.* [19] reported that a_w affected the regioselectivity in the preparation of diglycerides by *Penicillium roquefortii* lipase-catalyzed alcoholysis of triglycerides. The regioselectivity also increased with increasing a_w. The yield of diglycerides was 95% at high $a_w > 0.8$. Regioselective monoacylation of sucrose was performed using *Mucor miehei* [34]. The barium hydroxide, 8/1 H_2O (a_w=0.44) was selected for a suitable a_w buffer. Lysophospholipids were also synthesized using Lipozyme (Novo) [28]. Optimal a_w values for lysophosphatidic acid, lysophosphatidylethanolamine, and lysophosphatidylcholine were 0.18, 0.37, and 0.60, respectively, indicating that the more hydrophilic the substrate and reaction products, the higher the optimal a_w value. Similar result was shown in the lipase-catalyzed esterification of ethylene glycol to mono- and diesters [12]. In addition, an accurate control of a_w was essential for the high enantioselectivity in the lipase-catalyzed esterifications [4, 8, 31]. Hogberg *et al.* [30] reported the esterification of 2-methyloctanoic acid with an excess of *n*-dodecanol and *n*-tetra-decanols using *Candida rugosa* lipase in cyclohexane at 25°C without or with either $Na_2SO_4 \cdot 10/0$ H_2O (a_w=0.8) or $Na_2HPO_4 \cdot 2/0$ H_2O (a_w= 0.16), respectively. The esterification reactions were shown to be dependent on the a_w. Esterification without $Na_2SO_4 \cdot 10/0$ H_2O showed a rate profile with an induction period before the reaction rate increased. The apparent E-values were 23 at < 35% conversion. With the salt mixture ($Na_2SO_4 \cdot 10/0$ H_2O) present, the rate profile was much more linear up to close to 40% conversion, and the E-values at < 35% conversion were 91. However, Bovara *et al.* [10] showed the contradictory results in the transesterification of (±)-sulcatol with vinyl acetate using lipase from *Pseudomonas cepacia* and lipoprotein lipase from *Pseudomonas* sp. The results showed that variation of a_w markedly influenced the transesterification rate, but did not modify the enantioselectivity of the two enzymes. Similarly, Berglund *et al.* [3] studied the effect of a_w in the esterification of 2-methyldecanoic acid using lipase from *Candida rugosa*. The results showed that the enantioselectivity was independent of the a_w in the reaction medium using *n*-heptanol as the acyl acceptor. However, when *n*-decanol was used as the acyl acceptor, water activity in the reaction medium influenced the enantioselectivity. Therefore, in lipase catalysis, effects of a_w on enantioselectivity were varied with the kinds of substrate and lipase.

Methods for Measuring and Controlling Water Activity

Many reseachers have used various sensors in an equilibrium gas phase above organic solvents [5, 21, 22, 25]. The headspace in the gas phase normally contains significant amounts of the solvent vapor, which must have a deleterious effect on the sensor. In order to overcome the problem, Blanco, *et al.* [5] used the LiCl humidity sensor which endured organic solvents. Kahn and coworkers [33] developed an aluminum oxide a_w sensor which was able to be immersed in organic solvent. However, these a_w sensors still had the limitation of stability, sensitivity, and measurement range in organic solvents [33]. Instead of measuring a_w, methods of

adjusting and controlling water activity have been developed. Goderis *et al.* [20] first applied saturated salt solution to fix water activity of biocatalyst. Since his work, extensive studies have been made [1, 13, 22, 55]. Table 1 shows the a_w of saturated salt solutions arranged in increasing order of their a_w at 25°C. However, the preequilibration using saturated salt solution is impossible to maintain the a_w in lipase-catalyzed esterification because water is continuously formed during the reaction. Other methods were proposed when water was consumed or produced by the reaction. Goldberg *et al.* [21] used a silica gel as a water activity buffer. They measured the water content and a_w of the silica gel separately. Halling and Macrae [25] maintained the a_w by adding preequilibrated silica gel to the reaction mixture. However, the buffering capacity of the silica gel was difficult to be estimated, and the setting of the desired water activity was also difficult. Solid salt hydrate pairs have been applied to various enzyme reactions as a_w buffer [26, 28, 34, 36, 37, 38, 39, 65]. These give a perfect buffering capacity theoretically, and a_w remains completely constant unless the buffering capacity is deviated. The a_w of salt hydrate pairs and their buffering capacity are shown in Table 2. However, the salt hydrate pairs have the possibility of adverse effects on the biocatalyst, the reactivity of the salt with reaction substrates, the toxicity of the salt, and a difficult recovery and reuse of the salt and/or the enzyme in the economical operation of a large-scale reactor [47]. Recently, the effect of various types of solid salt hydrate pairs on acyl migration in 1,2-dibutyrin was investigated. The results show that the rate of acyl migration is faster when hydrogen phosphate salts are included compared to sulfate salts [50]. Also, transfer of the ions from the salt to lipase was reported [66]. In order to overcome the problems, various methods for controlling a_w were proposed. Zacharis *et al.* [66] investigated various salt hydrate pairs, and chose suitable salt hydrate pairs which are not deleterious to the lipase. Rosell *et al.* [47] developed a twin-core packed bed reactor for overcoming the problem. The method allowed the salt recovery and permitted a_w control without direct contact between immobilized lipase and salt. Various membrane reactors have also been developed for a_w control [40, 51, 58, 63]. Wehtje *et al.* [63] developed a method based on the gas phase equilibration by saturated salt solution. They used a silicone membrane for separating the reaction medium and biocatalyst from the salt solution. A saturated salt solution of known a_w was circulated slowly inside a silicone tube. The tube was submerged in the reaction medium. Water vapor can be transported through the tubing wall and the water activity in the reaction medium can thereby be continuously controlled. Similarly, pervaporation membrane was applied by van der Padt *et al.* [58]. They used a porous cellulose membrane. The air was recirculated through the reactor after condensing out the permeated water, and water activity could be kept constant by controlling the temperature of the condensor. The method, however, was applicable only to a solvent-free system. Lately, Kwon and Rhee [40] developed an on-off control system using a tubular type pervaporation membrane for a_w control. This method made it possible to carry out the lipase-catalyzed esterification in organic solvent with a continuous a_w control. Hollow fiber membrane reactors for a_w control have been developed [48, 53]. Packed bed hollow fiber membrane reactors were used for the lipase catalysis in organic phase at constant a_w [48]. *In situ* water activity control was carried out by pumping saturated salt solutions through the microporous polypropylene hollow fiber membranes. Similarly, Ujang *et al.* [53] developed a gas-phase hollow fiber reactor (GPHFR). The reactor consists of the hollow fiber dialyzer modules with enzyme immobilized on the lumen of the hollow fiber membranes. A constant humidity gas phase circulated through the shell of the reactor to control a_w.

Table 1. Water activity of saturated salt solutions at selected temperatures [46].

Salt	Temperature (°C)							
	5	10	15	20	25	30	35	40
LiCl	0.16	0.14	0.13	0.12	0.11	0.11	0.11	0.11
CH_3COOK	0.25	0.24	0.24	0.23	0.23	0.23	0.23	0.23
$MgBr_2$	0.32	0.31	0.31	0.31	0.31	0.30	0.30	0.30
$MgCl_2$	0.33	0.33	0.33	0.33	0.33	0.32	0.32	0.31
K_2CO_3		0.47	0.45	0.44	0.43	0.42	0.41	0.40
$Mg(NO_3)_2$	0.54	0.53	0.53	0.52	0.52	0.52	0.51	0.51
NaBr	0.59	0.58	0.58	0.57	0.57	0.57	0.57	0.57
$CuCl_2$	0.65	0.68	0.68	0.68	0.67	0.67	0.67	0.67
CH_3COOLi	0.72	0.72	0.71	0.70	0.68	0.66	0.65	0.64
$SrCl_2$	0.77	0.77	0.75	0.73	0.71	0.69	0.68	0.68
NaCl	0.76	0.75	0.75	0.75	0.75	0.75	0.75	0.75
$(NH_4)_2SO_4$	0.81	0.80	0.79	0.79	0.79	0.79	0.79	0.79
$CdCl_2$	0.83	0.83	0.83	0.82	0.82	0.82	0.79	0.75
KBr		0.86	0.85	0.84	0.83	0.82	0.81	0.80
Li_2SO_4	0.84	0.84	0.84	0.85	0.85	0.85	0.85	0.81
KCl	0.88	0.87	0.87	0.86	0.86	0.84	0.84	0.83
K_2CrO_4	0.89	0.89	0.88	0.88	0.87	0.86	0.84	0.82
C_6H_5COONa	0.88	0.88	0.88	0.88	0.88	0.88	0.86	0.83
$BaCl_2$	0.93	0.93	0.92	0.91	0.90	0.89	0.88	0.87
KNO_3	0.96	0.95	0.95	0.94	0.93	0.92	0.91	0.89
K_2SO_4	0.98	0.97	0.97	0.97	0.97	0.97	0.96	0.96
Na_2HPO_4	0.98	0.98	0.98	0.98	0.97	0.96	0.93	0.91
$Pb(NO_3)_2$	0.99	0.99	0.98	0.98	0.97	0.96	0.96	0.95

Conclusion

Lipases have moved from their original applications to the biotechnological processing of fats and oils. Recently, lipases have been widely applied in the area of organic chemistry [2, 18, 43, 49]. In particular, the production of

Table 2. Water activity values and their buffering capacity of various salt hydrates [26].

Salt pair	Temperature (°C)									Buffering capacity (mmol H_2O/g)	Max. Temp. (°C)
	20	25	30	35	40	50	60	70	80		
Na_2SO_4 (10/0)	**0.76**	**0.80**	**0.83**	×	×	×	×	×	×	31	32
Na_2HPO_4 (12/7)	**0.74**	**0.80**	**0.85**	0.90	×	×	×	×	×	14.0	35
Na_2CO_3 (10/7)	**0.72**	**0.75**	**0.79**	×	×	×	×	×	×	10.5	32
Na_2CO_3 (7/1)	0.66	0.70	0.74	0.78	×	×	×	×	×	26	35
$Na_2B_4O_7$ (10/5)	0.58	0.61	0.65	0.69	*0.72*	*0.80*	-	×	×	13.1	60
Na_2HPO_4 (7/2)	**0.57**	**0.61**	0.65	0.69	0.73	×	×	×	×	18.6	48
$ZnSO_4$ (6/1)	**0.55**	**0.59**	**0.62**	**0.66**	**0.69**	×	×	×	×	18.6	49
$Na_4P_2O_7$ (10/0)	0.46	0.49	0.52	**0.56**	**0.59**	**0.67**	**0.75**	**0.83**	0.92	22	80
NaBr (2/0)	**0.33**	**0.35**	**0.38**	**0.41**	**0.43**	**0.49**	×	×	×	14.4	50
$CuSO_4$ (5/3)	0.30	**0.32**	**0.35**	**0.38**	**0.41**	**0.48**	**0.55**	**0.63**	0.72	8.0	96
$Ba(OH)_2$ (8/1)	0.28	0.31	0.34	0.37	0.40	*0.47*	*0.54*	-	-	22	>100
CH_3COONa (3/0)	*0.25*	*0.28*	*0.30*	*0.32*	*0.35*	*0.41*	×	×	×	22	58
Na_2CO_3 (1/0)	0.22	0.24	0.27	0.29	0.32	0.38	0.44	0.51	0.59	8.1	110
$BaCl_2$ (2/1)	*0.20*	*0.23*	*0.25*	0.28	0.30	0.36	0.43	0.50	0.58	4.1	102
$CuSO_4$ (3/1)	0.196	0.22	**0.24**	**0.26**	**0.28**	**0.33**	**0.39**	**0.45**	**0.52**	9.4	>100
$MgHPO_4$ (3/1)	-	-	-	-	*0.26*	*0.25*	*0.25*	*0.25*	*0.24*	11.5	>100
Na_2HPO_4 (2/0)	0.150	0.163	0.177	*0.191*	*0.21*	-	-	-	-	11.2	95
$BaBr_2$ (2/1)	0.139	0.156	0.174	0.193	0.21	0.26	0.31	0.37	0.44	3.0	113
NaI (2/0)	*0.111*	*0.121*	*0.130*	0.141	*0.151*	*0.174*	*0.198*	×	×	10.8	68
Li_2SO_4 (1/0)	-	*0.095*	*0.101*	0.108	*0.114*	*0.128*	*0.143*	*0.159*	*0.175*	7.8	233
$CaCl_2$ (2/1)	0.037	0.040	0.043	0.046	0.049	0.055	0.062	0.069	0.076	6.8	170
$MgCl_2$ (6/4)	0.036	0.039	0.041	0.043	0.046	0.050	0.056	0.061	0.066	9.8	117
LiCl (1/0)	*0.017*	*0.020*	*0.023*	*0.026*	*0.029*	0.037	0.047	0.058	*0.071*	16.6	>90
$MgCl_2$ (4/2)	0.01 to 0.02 throughout									12.0	182

Hydrate forms are identified simply by giving the number of water molecules, so that, for example, "$MgCl_2$ (6/4)" refers to a mixture of $MgCl_2 \cdot 10H_2O$ and $MgCl_2 \cdot 4H_2O$. These are the smoothed, interpolated, or extrapolated values obtained from equation $\log a_w = A - B/t$ (where A and B are characteristic constants, T in Kelvin). The values considered most reliable are in **bold**, those least reliable in *italics*. The mark (-) indicates that the value at this temperature is considered too uncertain to quote. The mark × indicates that the higher hydrate involved is probably or certainly unstable at this temperature, so the pair should not be used. The maximum temperature above the higher hydrate form is known to be unstable.

the enantiomerically pure compounds is very important in the application of lipases because the portion of the chiral pharmaceuticals introduced as single enantiomers will grow very rapidly in the future [43]. In these reactions, water activity control is essential for the high production yield. Many reseachers have developed the methods for controlling a_w in the lipase catalysis. However, some problems still remain in a large-scale production. Therefore, convenient methods for controlling a_w will be required for a large-scale reactor for the high production yield of various useful compounds, such as chiral drugs, flavors, dietary lipids, and emulsifiers.

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