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Dependency of Water Availability on the Esterifying Activity of *Candida cylindracea* Lipase in Organic Solvent

NOOR, IZANI NOOR JAMIL AND IBRAHIM CHE OMAR*

Fermentation and Enzyme Technology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. Minden, 11800, Penang, Malaysia

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Abstract To establish optimal conditions for esterification by *Candida cylindracea*, lipase reactions were performed simultaneously, separately, or individually in the varying initial rates of 0.014–0.060 μmole free fatty acids consumed min^{-1} g^{-1}. The reactants which were conditioned at a_w of 0.12 gave the highest initial rate of esterifying 0.060 μmole free fatty acids consumed min^{-1} g^{-1}. These results suggest that the esterifying activity of lipase in an organic system depends on the transfer of available water within the reaction system.

Key words: Conditioning of reaction mixture, esterifying activity, *Candida cylindracea* lipase, organic solvent

Studies on an enzyme activity in the media of low water content have shown that the catalytic activity can be maintained at optimum water activity (a_w) in the enzyme system [4, 11, 16]. The optimal amount of water is bound to the enzyme, and a suitable solvent must be chosen so as not to strip water off the enzymes [2, 17]. Nevertheless, the bound water is actually in a very dynamic state which depends on the transfer of water between the phases in the reaction system. The water transfer is inevitable in the reaction system consisting of enzymes, reactants, and solvents having different degrees of hydrophobicity and water adsorptivity until the system reaches a state of equilibrium [9, 15]. All the reaction components can affect the relationship between water and the catalyst through a simple competition for the available water, but this is not an interaction at the molecular level between the biocatalysts and the reaction components with water. It merely relies on the water binding capacity; high water binding capacity of a substrate may result in a lower biocatalytic activity. The rate of water transfer will remain low during the reaction if the reaction components have been equilibrated to the reaction water activity. However, when the components exhibit large variation in the a_w, the rate of water transfer within the phases varies significantly and, thus, the biocatalytic activity becomes very unstable and unpredictable. The biocatalytic activity can be improved by optimizing the reaction system at the respective a_w. This paper describes the variation in the esterifying activity of lipase of *Candida cylindracea* after conditioning the reaction components at different a_w using saturated salt solutions.

The crude lipase of *Candida cylindracea* Strain OF (Meito Sangyo, Japan) with an activity of 43,000 U/g powder was used throughout the experiments. One unit of lipase is the amount of enzyme needed to liberate 1 μmol of free fatty acid per min [7]. The reaction mixture for butyl oleate synthesis consists of 0.32 ml oleic acid (0.2 M) and 0.37 ml n-butanol (1.0 M) in 5 ml of water saturated n-hexane. The reaction was carried out at 37°C with agitation of 200 rpm for 1 h in a 50-ml conical flask, screw-capped to prevent evaporation of the solvent. The reaction was initiated by adding 10 mg of enzyme powder which was dried under molecular sieve pellets (4 Å alumino borosilicate - Fluka, Switzerland) prior to use. All the reactants and solvents were kept in the presence of molecular sieve pellets to keep the water content of the reaction components to a minimum. The saturated salt solutions used and their respective a_w [3] were LiCl (0.12), CH_3COOK (0.22), K_2CO_3 (0.44), NaBr (0.55), KI (0.69), NaCl (0.76), $(NH_4)_2SO_4$ (0.81), KCl (0.86), KNO_3 (0.92), and K_2SO_4 (0.97). Pre-equilibration was performed for 48 h in a closed container containing the salts, the reaction mixture, or the individual reaction components [5]. The water content after pre-equilibration was then determined. The pre-equilibration procedures were meant to condition the reaction mixture with respect to the a_w of the saturated salt solution.

Five different pre-equilibrations were performed to condition the reaction mixture. The first system performed was simultaneous conditioning, which refers to the pre-equilibration of the entire reaction mixture in the absence of enzyme preparation. The reaction was initiated by the

*Corresponding author
Phone: 60-4-6577888; Fax: 60-4-6565125,
E-mail: oibrahim@usm.my

addition of the enzyme preparation. The second system was the pre-equilibration of each of the reaction components separately. The reaction was initiated by mixing the reaction components and the enzyme preparation. In the third, fourth, and fifth systems, the enzyme preparation, *n*-hexane, or the substrate (oleic acid and *n*-butanol) were singly pre-equilibrated, while the other components of the reaction mixture were not conditioned. After the pre-equilibration of one of the reaction components, it was added to the reaction mixture to initiate the reaction. Water content in all the reaction systems was determined. The esterifying activity (synthesis of butyl oleate) was measured by the amount of oleic acid consumed for the synthetic reaction. The esterifying activity of the enzyme was expressed as the initial rate based on the fatty acid consumed for the reaction (μmole oleic acid consumed min^{-1} g^{-1} enzyme). The acid was determined by the method of Lowry and Tinseley [10]. The water content in the organic phase of the reaction system was determined by the Karl Fischer Titration (Metrohm, Switzerland).

Figure 1 shows the water adsorption isotherms of each reactant at different a_w of the saturated salt solution. The substrate, *n*-butanol, exhibited a rapid adsorption rate with a maximum water content of about 8% at a_w 0.97. Enzyme preparation and other components exhibited maximum water adsorptions ranging from 0 to 3% (w/w). The figure clearly shows that the nature of hydrophilicity and hydrophobicity of the reactants may act as determinants for the reaction rate as they actually determine the level of water within the system. Therefore, in the reaction system which contains both the hydrophilic and hydrophobic reactants, the extent of conversion tends to fluctuate, resulting in an unstable conversion degree as shown in the glycerolysis reaction [6]. However, if the a_w of each reaction component can be fixed, the profile of the biocatalytic activity will be the sole function of the water adsorption isotherm of these components. This was done through the conditioning of the reaction system using different salts with different a_w to illustrate the requirement

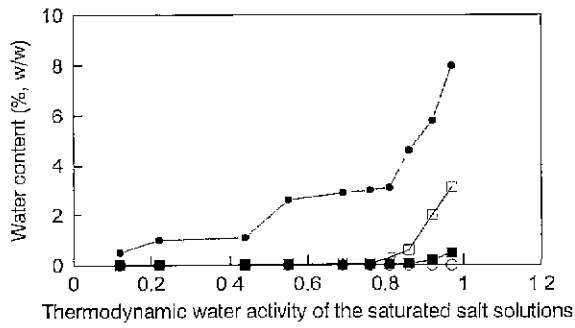

Fig. 1. Water adsorption isotherms of the reaction components at different a_w.
Symbols. (○) *n*-hexane, (●) *n*-butanol, (□) enzyme preparation, and (■) oleic acid

of an optimum water content within the system. The water adsorption isotherms will also give information on the water budget within the system. It is also noted that water is formed during esterification as well which constitutes a fraction of the total water in the system.

Figure 2 shows the profile of the esterifying activity and the water content in the system which was conditioned simultaneously at different a_w. The optimum esterifying activity was obtained at a_w of 0.44. The water content profile dropped from the a_w of 0.12 to 0.44 and increased gradually up to the a_w of 0.97, which subsequently resulted in a rapid drop in the esterifying activity. In this system, the water adsorptivity of the reaction components will determine the water content for each component until an equilibrium state for all the phases has been achieved. The competition for the available water amongst the reaction components is possibly involved in this system. The outcome of the competition is governed by the a_w of the system. In this system, the competition will take the form of water equilibrium between the phases in response to the a_w used for the conditioning. The rate of the esterifying activity now correlates to the time required to achieve equilibrium at the a_w of the system.

In the case of the pre-equilibration of the reaction components which was done separately, each reaction component contained different amounts of water although at the same a_w (Fig. 2). The rapid drop in the initial rate of esterification correlated with the increase in water content in the organic phase as the a_w of the saturated salt solutions increased. This system did not allow a stable equilibrium to be achieved, as the large quantity of water mainly in the enzyme preparation and the reactants was not effectively transferred. At higher a_w, the enzyme particles tended to aggregate and no catalytic activity was observed.

The enzyme preparation which was pre-equilibrated under different a_w showed an optimum esterifying activity at a_w of 0.44. with a maximum reaction rate of 0.033 μmole

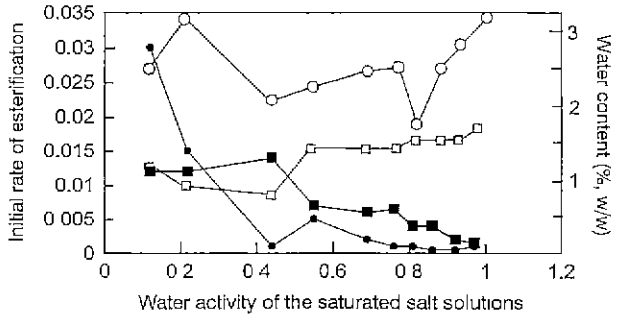

Fig. 2. Effect of simultaneous and separate conditioning of the reaction mixture on the esterifying activity and water content at different a_w.
Symbols: (●, ○) simultaneous conditioning. (■, □) separate conditioning (closed symbols) initial reaction rate, (open symbols) water content in organic phase

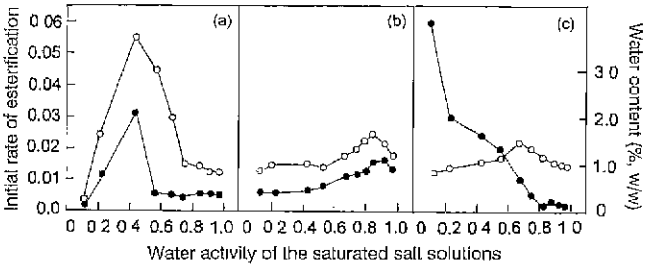

Fig. 3. Effect of conditioning of enzyme preparation, n-hexane and the reactants. (a) Enzyme preparation, (b) *n*-hexane, (c) reactants.
Symbols: (●) initial reaction rate, (○) water content in organic phase

of acid consumed min^{-1} g^{-1} (Fig. 3a). A similar profile was observed with the water content in the system. The water taken up by the enzyme upon pre-equilibration using the saturated salt solution of a_w up to 0.44 was readily transferred to a polar reactant maintaining adequate water amount for biocatalysis. At high a_w, the enzyme particles tended to form strong aggregates with water during pre-equilibration which resulted in the limitation of the interfacial area for water transfer.

Water adsorption by more polar solvents is higher compared to less polar solvents, depending on the water miscibility in these solvents. In the case of *n*-hexane (Fig. 3b), it was observed that the maximum esterifying activity was at the a_w of 0.92, which thereafter decreased. In this experiment, water was made available in the organic phase for the reaction which was transferred to the other reaction components. Valivety *et al.* [13] reported that the lipase activity was optimum at a_w 0.55, although different organic solvents of varying polarity from *n*-hexane to pentanone were used. Although there were differences in the reaction rate indicating some form of solvent dependency, the general reaction profile remained unchanged. The water in the organic phase seems to determine the esterifying activity, as it determines the water present around the biocatalyst particles and in the substrates.

n-Butanol is a hydrophilic substrate which possesses high water binding capacity. Competition for the water affects the water concentration in the polar reactant, and its concentration should be similar to those with the altered bulk phase in the solvent [8]. Goldberg *et al.* [1] reported a higher competition for water for transesterification with propanol than with heptanol. Upon pre-equilibration, the rate of esterification was maximum at a_w of 0.12 with the rate of 0.060 μmole of free fatty acid $min^{-1}g^{-1}$. However, a rapid drop was observed as the a_w of the saturated salt solution increased (Fig. 3c). This profile resembled the system that was conditioned separately (Fig. 2b). This coincided with the rapid increase of the water content, as the substrate adsorbed the water under increasing a_w of the saturated salt solutions. Therefore, at constant water activity, the system would be in suboptimal hydration. Norin *et al.* [12] observed a drop in the esterifying activity with an increase in acid concentration due to water solubility.

Table 1 shows the summary of the initial rate of esterification under different conditioning procedures. Conditioning of the reactants at the a_w of 0.12 gave the highest reaction rate of 0.06 μmole of free fatty acid min^{-1} g^{-1} compared to the other procedures. With an exception to the pre-equilibration of only the enzyme, the initial rates significantly correlated with the water present in the organic phase.

In conclusion, the results obtained in the work demonstrate that the rate of esterification in organic solvent depends on the water made available to the system. The water is transferred within the system, depending on the hydrophobicity and hydrophilicity of the reaction components. Water will be dissolved in the solvent phase or bound to the biocatalyst and the reactants. Water can also be transferred to and from the gas headspace. The water transfer rate between these phases will be directed to achieve the state of equilibrium in the system. The time taken to achieve the equilibrium state can be considered as the lag phase of the reaction, which means that the longer lag phase is an indication of the lower initial rate observed.

In the reaction system in which it is simultaneously conditioned at a fixed a_w, a state of equilibrium has priorily been achieved. Therefore, there will be no lag phase and the reaction rate depends entirely on the rate of water transfer to the enzyme by the system. However, if these

Table 1. Comparison of the conditioning procedures of the reaction systems on the maximum initial rate of esterification

Conditioning procedures	Optimum a_w of saturated salt solutions	Water content in organic phase (%, v/v)	Initial rate of esterification (μmole of free fatty acid $min^{-1}g^{-1}$)
Control*		0.01	0.0085
Simultaneously	0.44	2.15	0.014
Separately	0.12	1.05	0.030
Enzyme	0.44	3.55	0.033
Solvent (*n*-Hexane)	0.92	1.50	0.017
Reactants (oleic acid and *n*-butanol)	0.12	0.95	0.060

*All the reaction components were kept in the presence of molecular sieve pellets

components are equilibrated separately or individually, the water mediation is multidirectional: that is, from the water source to the respective reaction components. The hydration state of the reaction components may be optimal or sub-optimal depending on the water binding capacity of the reaction components. Under these conditions, water becomes relatively competitive, resulting in the variation of the lag phase of the reaction and subsequently changing the initial rate of the esterification reaction.

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