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SHORT COMMUNICATION

Some characteristics of Amberlite XAD-7-adsorbed lipase from *Pseudomonas* sp. AK

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Immobilization of lipases using various support materials has been reported extensively (Ibrahim *et al.* 1988) which is used for a number of applications such as hydrolysis, esterification and transesterification. Reports on the application of lipases for the synthesis of acetone glycerol acyl esters were also available. However, most of the reactions were catalysed by the immobilized lipase of *Mucor meihei* on resins (Lipozyme™, Novo Nordisk) (Ibrahim *et al.* 1989, Pecnik and Knez, 1992). Hess *et al.* (1995) also reported the use of *Pseudomonas cepacia* for the synthesis of monostearoyl glycerol from 1,2-*O*-isopropylidene glycerol. Nevertheless, these lipases exhibited low stability in organic solvents resulting in poor conversion rate. Therefore, alternative lipase preparations for the synthesis of acetone glycerol acyl ester with high stability and conversion degree must be sought which can be used for continuous biocatalysis in bioreactor systems. Acetone glycerol acyl esters are the intermediates for the formation of monoglycerides via hydrolysis using concentrated hydrochloric acid in cold diethyl ether solution. The advantageous point of monoglyceride production via acetone glycerol acyl esters resulted in the production of almost 100% yield of the monoglycerides. Monoglycerides are widely used as emulsifiers and stabilizers in food industry. It can also be produced by the lipase catalysed hydrolysis of triolein (Plou *et al.* 1996). Although the efficiency of the hydrolysis was considered significant, the final products consisting of a mixture of diolein and monoolein necessitates the separation stage. At the same time, monoolein and diolein are molecularly unstable and prone to spontaneous isomerisation resulting in low yields of the desired monoglycerides (Ibrahim *et al.* 1987).

In the search for enzyme preparation with high conversion degree coupled with high stability in organic solvent, this short communication highlights the selection and some characteristics of Amberlite XAD-7-adsorbed lipase which will be used for the formation of monoglycerides via acetone glycerol acyl esters.

All the crude lipase preparations used in the work were generous gift of Amano Pharmaceutical Co. Ltd (Nagoya Japan). The enzyme preparations were used without prior purification. The lipase hydrolytic activity of each preparation were determined based on the method of polyvinyl alcohol - olive oil emulsion method described by Ibrahim *et al.* (1987). The fatty acids liberated from the hydrolysis of olive oil was titrated against 0.05 N NaOH at the end point of pH 9.0 using a pH stat (Titramate Mettler Toledo). One unit (U) of lipase is defined as the amount of enzyme required to liberate 1 μmole of free fatty acid per min, at the respective temperature and pH as documented by Amano Pharmaceutical Co. Ltd.

Immobilization of lipase was carried out by the adsorption method as described by Ibrahim *et al.* (1988) with modifications in the immobilization conditions based on the characteristics of the lipase of *Pseudomonas* sp. used. The support materials used consist of Celite 545 (Nacalai Tesque), Amberlite XAD-4, Amberlite XAD-7 (Fluka Chemika), Kiselguhr (Merck), Florisil (Merck), Eupergite C and Eupergite C250L (Rohm Pharma). The immobilization was carried out by dissolving 0.5 g of enzyme powder in 50 ml of McIlvaine buffer of pH 5.0.

The undissolved materials were decanted by filtration using Whatmann No 1 filter paper. The filtrate was added with 1.0 g of the support material and agitated at 100 – 150 rpm, at 32 – 37°C for about 50 – 120 mins. After the agitation, the absorbed lipase was crosslinked with glutaradehyde at the final concentration of 2.5% (v/v) for 60 min. The adsorbed and cross linked lipase was filtered and washed thrice with distilled water and dried overnight at room temperature before the activity was determined. The immobilization yield (%) was given by the ratio between the total activity of the immobilized preparation and the total activity of the lipase used for immobilization. The reaction for the synthesis of acetone glycerol esters was carried out in a screw-capped bottles consisting of 1,2-*O*-isopropylidene glycerol at 8 mM, lauric acid at 8 mM, 10 mg enzyme powder for the free lipases and 100 mg for the immobilized lipases in 10 ml of n-hexane. The reaction was carried out at 32°C with shaking of 150 rpm for 24 hr. Product formation was analysed using gas chromatography (Shimadzu GC 14A). The column used for the analysis was Permabond SE-54-HKW-1 (Macherey and Nagel, Duren, Germany) of size 0.32 mm internal diameter and 25 m in length. The analysis conditions were carrier gas hydrogen (1 kg/m^2) and nitrogen (0.3 kg/m^2) with the flow rate of 2.0 ml min^{-1}, injection temperature of 280°C, detector temperature of 280°C, temperature

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programme 250°C (2 min) → 280°C (15 min) and injection volume of 0.1 µl. The ester produced and the residual reactants were confirmed with Thermofinnigan Trace gas chromatography mass spectroscopy (GCMS) using the conditions of carrier gas helium (50 psi) and nitrogen (0.3 atm) at the flow rate of 1.0 ml min^{-1} with injection temperature of 250°C and injection volume 0.1 µl. The conversion degree was calculated based on the composition weight % of the esters and reactants.

Figure 1 shows the results of the rate of 1,2-isopropylidene-lauroyl glycerol synthesis by various free lipase preparations. As shown in the figure, the lipase from *Pseudomonas* sp. AK was found to show the highest rate of ester synthesis of 11.43 weight % per h reaction time. Other lipases showed varying degree of the conversion rate with significant activities with lipases of *Mucor javanicus, Thermomyces lanuginosus, Penicillium camembertii* and *Candida cylindracea* in the range 5.0 – 5.7 weight % per hr reaction time. *Aspergillus roquefortii* or *Aspergillus niger* were not suitable for the synthesis of 1,2-isopropylidene-lauroyl glycerol. Thus, the lipase of *Pseudomonas* sp. AK was selected to be used for the immobilization experiments.

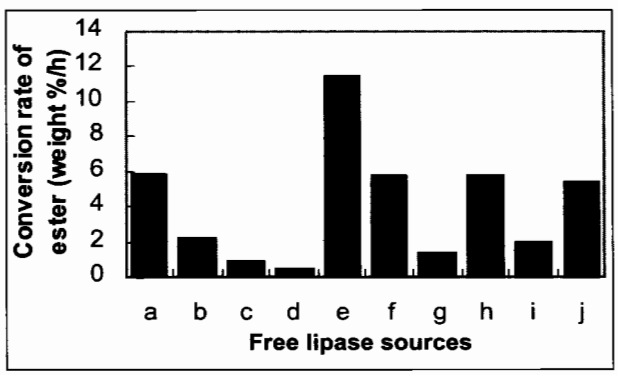

Figure: 1 Conversion rate of 1,2-isopropylidene lauroyl glycerol by various free lipases.
a : *Candida cylindracea*, b: *Rhizopus niveus*, c: *Penicillium roquefortii*, d: *Aspergillus niger*, e: *Pseudomonas* sp. AK, f: *Thermomyces lanuginosus*, g: *Candida rugosa* AY, h: *Penicillium camembertii*, i: *Rhizopus oryzae*, and j: *Mucor javanicus*.
All data shown were mean of triplicates experiments

Figure 2 shows the immobilization yield of lipase from *Pseudomonas* sp. adsorbed on various support materials. Under the optimized adsorption conditions of pH 5.0, temperature 32°C, agitation rate 150 rpm, and adsorption time 120 min, the highest immobilization yield of 48.5% was obtained when Amberlite XAD-7 was used as the support material. Other support materials show lower immobilization yield of less than 40%. Although the immobilization yield of *Pseudomonas* sp. adsorbed on the Amberlite XAD-7 was moderate of 48.5%, however the conversion degree of 68.2% was considered significant. Celite also gave a signification conversion degree of 56.5%. On the other hand, lower or no esterification occurred when adsorbed to either Amberlite XAD 4, Kieselguhr, Florisil or Eupergites, although these preparations exhibited enzyme activity under aqueous conditions. Noor Izani and Ibrahim (1997) and Norin *et al.* (1988) have shown that the hydrophobicity of the support materials which determines their ability to maintain the essential water monolayer and subsequently maintaining the enzyme integrity and activity in organic solvent biocatalysis. The lipase adsorbed to Amberlite XAD-7 was further used in subsequent experiments.

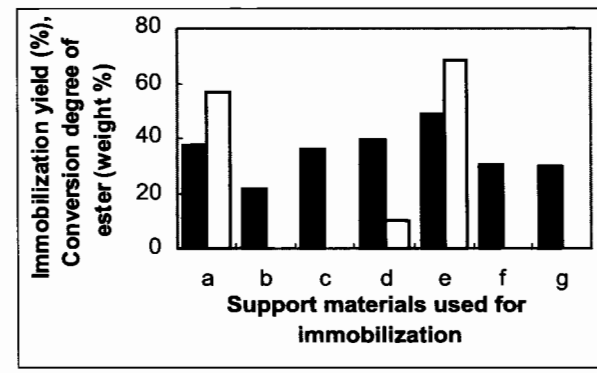

Figure: 2 Immobilization yield and conversion degree by lipase of *Pseudomonas* sp. on various support materials.
a: Celite 545, b: Florisil, c: Kieselguhr, d: Amberlite XAD-4, e: Amberlite XAD-7, f: Eupergite C and g: Eupergite C250L. All data shown were mean of triplicates experiments
(■) Immobilization yield (□) Conversion degree of esters.

Some of the characteristics of the Amberlite XAD-7-adsorbed lipase with respect to the effect of pH and temperature on the hydrolytic activity were examined. Comparison was made between free and immobilized lipase preparations. The results obtained indicated that the immobilized lipase preparation showed a wide range of pH stability of 4.0 – 8.0 maintaining 100% activity (Figure 3). However, free lipase showed a narrower pH range of 6.0 – 8.0.

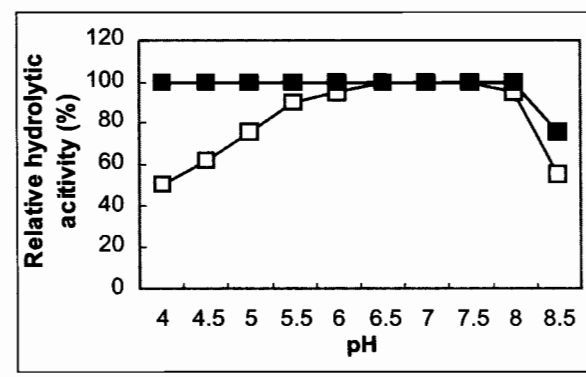

Figure: 3. Effect of pH on the hydrolytic activity of lipase from *Pseudomonas* sp. AK.
Buffer systems: Acetate buffer (pH 4.0 – 5.5), phosphate buffer (pH 6.0 – 8.5). Relative activity was calculated based on the activity of the lipases at pH 5.0. All data shown were mean of triplicates experiments.
(■) Amberlite XAD-7 adsorbed lipase (□) Free lipase.

The effect of temperature on the hydrolytic activity of free and immobilized lipases was found to show similar profiles (Figure 4). An optimum temperature of 55°C and the temperature stability of the free and immobilized lipases were in the range of 30 - 60°C maintaining 100% of the activity. Numerous reports have shown that immobilization may results in the shift of temperature effect when compared to its free state (Montero *et al.* 1993).

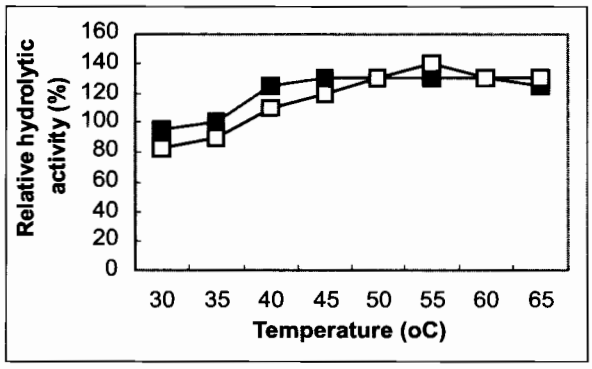

Figure: 4: Effect of temperature on the hydrolytic activity of lipase from *Pseudomonas* sp. AK
Relative activity was calculated based on the activity of the lipases at temperature 32°C. All data shown were mean of triplicates experiments
(■) Amberlite XAD-7 adsorbed lipase (□) Free lipase.

The stability in organic solvents by the free and immobilized lipases preparations was carried out by incubating 1 g of the immobilized preparation in 10 ml of either hexane, heptane or isooctane for an incubation time of 10, 20 and 30 h at 32°C and agitation of 150 rpm. After the incubation, the lipases were filtered and dried overnight under vacuum before the hydrolytic activity was determined by the PVA – olive oil emulsion method. The results shown in Figure 5 indicated that the free lipase showed too low or no hydrolytic activity after incubation in the organic solvents, however the immobilized lipases were found to be stable even after 30 h of incubation maintaining 100% of the synthetic activity in either n-hexane, heptane or isooctane. Noor Izani and Ibrahim (1998) have shown that apolar organic solvents with high Log P values were able to restore the enzyme activity effectively after rehydration. The results suggested that the high stability of the immobilized lipase preparation in organic solvent is vital in ensuring high bioconversion in organic synthesis. The optimization of the synthesis of 1,2-isopropylidene lauroyl glycerol catalysed by Amberlite XAD-7 adsorbed lipase of *Pseudomonas* sp. has been performed and a conversion degree of almost 100% was obtained. The results of these findings were reported elsewhere.

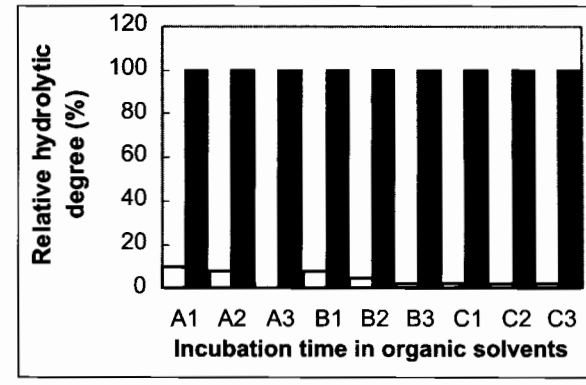

Figure: 5 Stability of hydrolytic activity of lipase of *Pseudomonas* sp. AK. in organic solvents with incubation time.
A1: 10 hr in hexane, A2: 20 hr in hexane, A3: 30 hr in hexane, B1: 10 hr in heptane, B2: 20 hr in heptane, B3: 30 hr in heptane, C1: 10 hr in isooctane, C2: 20 hr in isooctane, C3: 30 hr in isooctane.
Relative activity was calculated based on the activity of the lipases before incubation in the organic solvents. All data shown were mean of triplicates experiments

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