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THE CATALYTIC PROPERTIES OF HTAB-ALKANOL-HYDROCARBON-WATER MICROEMULSION SYSTEM FOR ESTERIFICATION

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Abstract: Microemulsions have been widely reviewed, not only as effective media for enzyme reactions requiring hydrophobic solvents but have also attracted wide interest as possible low-toxicity, low-cost media for tertiary oil recovery, formation of nanoparticles and decomposition of toxic pollutants. These fluids can be tuned for specific applications by adjusting their compositions. A water-in-oil (w/o) microemulsion system was developed in this study based on cationic surfactant, tert-amyl alcohol, aliphatic hydrocarbon and aqueous buffer. The microemulsion – HTAB/2M2B-Hexane/buffer – was then used as a reaction medium for lipase-catalysed esterification of sucrose and lauric acid. The synthetic activities of Lipozyme and Novozyme in the microemulsion were compared with activities in four other reaction media: hexane, isoocatane, tert-amyl alcohol (2M2B) and HTAB/2M2B/buffer. The microemulsion was found to be the best medium for the synthesis of sucrose lauryl esters, with Lipozyme showing higher activity compared to Novozyme. Lipozyme activity was found to increase as the incubation time increased; reverse effect was observed with Novozyme. Microemulsion system with 5/95 surfactant ratio and 5% water gave the highest yield of sugar ester by Lipozyme. This microemulsion can be proposed as a highly potential reaction medium for high productions of the industrially-important sugar ester.

KEYWORDS: w/o-microemulsion, cationic surfactant, lipase, sugar ester

Introduction

Water-in-oil microemulsions or reversed micelles, are being evaluated as a reaction medium for a variety of enzymatic reactions. These systems have many potential biotechnological applications. Important examples are the use of various lipase microemulsion systems for hydrolytic or synthetic reactions (Stamatis *et. al.,* 1999). The advantages of a microemulsion have also been employed in electrosynthesis instead of an organic solvent since it offers lower toxicity and cost, high dissolving power for reactants and mediators of unlike solubility, possible reaction pathway control and recycling of microemulsion components, contributing significantly to "green" electrochemical synthetic methods of the future (Rusling, 2001).

Microemulsions systems provide an enormous interfacial area through which the conversion of hydrophobic substrate can be catalysed since they consist of a bulk organic phase containing aqueous droplets stabilised by a surfactant. The inner cores of aqueous microphase are able to solubilise bio-products such as proteins (Gupta *et al.*, 2004). The biocatalyst remains soluble and active in the water, while substrates and products dissolve in the organic phase. The amount of aqueous phase is small, so that lipases can catalyse transesterification and ester synthesis reactions under these conditions (Ferrer *et al.*, 2002). The substrates can be enzymatically converted with high yields because the thermodynamic equilibrium of condensation/hydrolysis reactions can be easily shifted by adjusting the water content. Thus, microemulsions have been used as ideal media for lipase catalysis.

Sugar esters are well known as bio-surfactants. Their excellent biodegradability, odourless, tasteless, nontoxic, non-irritant and nonionic characteristics explain their increasing importance in numerous areas (Cao *et al.*, 1999). For a long time, the production of sugar ester remained mainly in the realms of organic chemistry and chemical processing. Chemical methods are mainly performed at high temperatures in the presence of alkaline catalysts. High-energy consumption, colouring of products and low selectivity are major disadvantages of these methods (Tarahomjoo and Alemzadeh, 2003). Moreover, some chemically-synthesised sugar esters are toxic and not readily biodegradable, thus causing their limited application in cosmetics, food industry and pharmaceuticals (Yan *et al.*, 1999). The application of enzymes to the synthesis of sugar ester has been actively studied due to the mild reaction conditions and high regioselectivity typically displayed by biological catalysts. Numerous studies have been reported using reverse micelles or microemulsions formed with anionic surfactants, most notably bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT), as confined spaces for nanoparticle formation. In comparison, there are relatively few studies using reverse micelles formed by cationic surfactants (Axnanda and Shantz, 2005). This paper reports the development of w/o-microemulsion using a cationic surfactant, hexadecyltrimethylammonium bromide (HTAB) and its use as a medium for lipase-catalysed sugar ester synthesis.

Materials and Methods

1) *Development of HTAB/Hexane-2M2B/Buffer microemulsion*

A preliminary investigation was carried out on the phase equilibrium between four component systems: hexadecyltrimethylammonium bromide (HTAB), hexane:2-methyl-2-butanol (2M2B; 50:50), and aqueous buffer. The HTAB and 2M2B were mixed at different weight ratios, to a total mass of 0.5g, and the aqueous buffer (20 mM Tris-HCl pH 7.0) was added drop by drop with gentle shaking after each drop until a clear solution was obtained. The mixing solutions were then centrifuged at 3200 rpm for 10 minutes and kept in a water bath at 30^{o}C for 5 minutes. After the desired equilibrium was reached, the sample mixtures were evaluated visually for clarity inspection between crossed polarisers, and under a polarising microscope for homogeneity and birefringency observation. The region of the phases: microemulsion (ME), liquid crystal (LC) and coarse emulsion (EM), can be estimated by noting the turbid-to-clear transitions and a phase diagram was then constructed.

2) *Lipase-catalysed esterification of sugar esters in different media.*

The esterification reactions of sucrose and lauric acid were carried out in 3 ml of the microemulsion and four other selected reaction media: hexane, isoocatane, tert-amyl alcohol (2M2B) and HTAB/2M2B/buffer. The enzymatic reactions were started by adding 100mg of immobilised lipase Lipozyme and Novozyme to a mixture of 50 mM of sucrose and 100 mM lauric acid. The reaction mixtures were incubated at 40^{o}C, 200 rpm for 24 hours. The amount of sucrose lauryl ester synthesised was measured in a spectrophotometric assay to indicate the activity of the enzymes.

3) *Assay of lipase activity.*

The rate of esterification activity by lipase was determined according to Kwon and Rhee (1999) with some modifications. The depletion of fatty acid was monitored by reading the absorbance at 715 nm. The amount of the free fatty acids left in the reaction mixture was quantified against a standard curve plotted for oleic acid.

Effect of incubation time on lipase-catalysed esterification in HTAB/Hexane-2M2B/Buffer microemulsion

Sucrose and lauric acid prepared in a molar ratio of 1:2 were mixed in 3 ml of microemulsion and 100 mg of immobilised lipase. The reactions were incubated at 40°C, 200 rpm for 3, 6, 24, 48 and 72 hrs. At stated time intervals, the reaction mixtures were aliquoted for lipase activity assay.

5) *Effect of surfactant ratio and water concentration of HTAB/Hexane-2M2B/Buffer microemulsion on lipase-catalysed esterification*

Various ratios of surfactant - 5/95, 10/90, 15/85, 20/80 - and different percents of water concentrations - 5%, 10%, 15% - were chosen to prepare the microemulsions. The amounts of sucrose, lauric acid and lipase in the reaction mixtures were kept constant. Syntheses of sugar esters were performed in the microemulsions at 40°C, 200 rpm for 24 hrs and lipase activity was then measured.

6) *Characterisation of sugar ester*

For product separation, the synthetic mixtures were filtered and precipitated using 10 ml of aliphatic alcohol. Ethyl acetate of approximately 10 ml was added to the precipitate, followed by two volumes of water in a separation funnel. The mixture was stirred for 5 min, left for a few minutes and the upper layer was taken out. The separated product was evaporated under vacuum and dissolved with a small amount of chloroform. Purification of ester product was carried out using silica gel column chromatography and eluted with chloroform/methanol (90/10 v/v), followed by evaporation of solvent and keping at 4°C overnight prior to analysis.

For product identification, Infra-Red Spectroscopy (IR) was performed. IR: 3456 ν(OH); 2923 ν(C-H); 1738 ν(C=O) and 1217 ν(C-O).

Results and Discussion

1) *Phase diagram system of HTAB/ Hexane-2M2B/Buffer*

Figure 1 shows the existence of two regions of homogeneous isotropic solution. One is in high aqueous content and the other is in high alcohol content, designated as $\mathbf{L}_1$ and $\mathbf{L}_2$ respectively. The limits of these areas were determined by titrating to turbidity with smallest amount of one component to the homogeneous solution (Hamdan *et al.*, 1993). It is clear that the areas of microemulsion range from w/o to bicontinous and to o/w, continuously over a wide water content in all phase diagrams. All mixtures produced optically-clear solutions at low water concentrations, forming the reverse micelle ($\mathbf{L}_2$). The titration was continued in order to observe the presence (or absence) of the second clear region ($\mathbf{L}_1$). The transition from clear to cloudy solution indicated that the maximum water solubilisation had been exceeded.

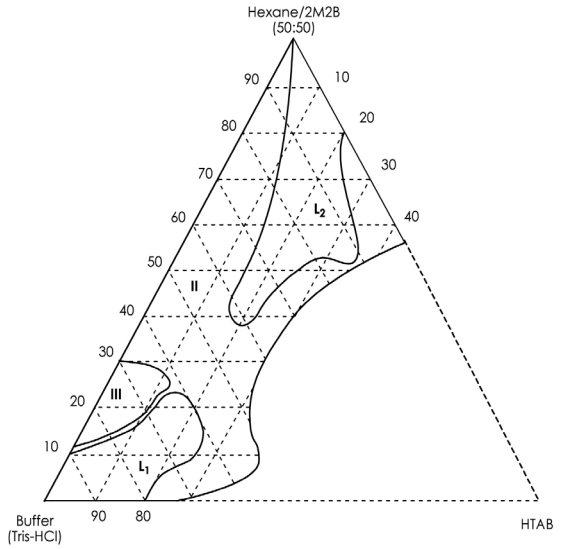

Figure 1: Phase diagram of microemulsion system of HTAB/Hexane-2M2B/Buffer. $\mathbf{L_1}$: oil-in-water system (1 phase); $\mathbf{L_2}$: water-in-oil system or reverse micelle (1 phase); **II** & **III**: emulsion system (2 or 3 phases).

Enzymatic synthesis of sugar esters in HTAB/Hexane-2M2B/Buffer microemulsion

In order to determine the efficiency of the microemulsion system for sugar ester synthesis, reactions were carried out and compared with four other media. A suitable organic solvent in which both substrates were best dissolved and reacted was identified.

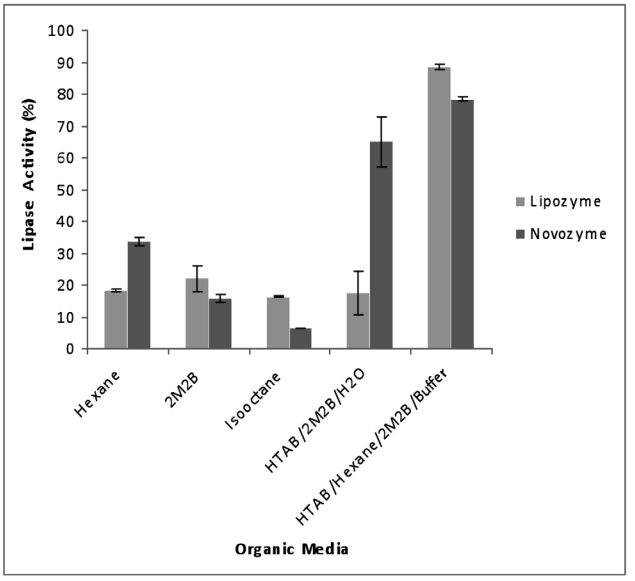

Figure 2: Lipase activity in HTAB/Hexane-2M2B/Buffer microemulsion in comparison with different organic media.

Figure 2 shows the result for the effect of five different types of organic media used in the enzymatic synthesis at 40°C for 24 hrs. The microemulsion HTAB/Hexane-2M2B/buffer shows the highest result with 88.3% activity for Lipozyme and 78.3% for Novozyme in the synthesis of sugar esters. This is expected as the major attraction of w/o microemulsion procedure is that the enzyme is dispersed at the molecular level. In such a system, enzymes are active as regards the conversion of both hydrophilic and hydrophobic compounds (Zoumpanioti *et al.*, 2006) while other emulsions forms the hydrophobic interior where substrate cannot enter entirely due to the limitation of dispersion, which will lead to lower conversion. According to Carvalho and Cabral (2000), there are many advantages of microemulsion: formation of spontaneous reversed micelles and therefore reaching an equilibrium state in a short time; solubilisation of both hydrophilic and hydrophobic substrates/products; synthetic processes are favoured due to the shift of thermodynamic equilibrium; increased interfacial area of contact (10–100 m^2/mL); improved activity/stability and enzyme aggregation is avoided.

For the effect of incubation time, synthetic reactions in w/o-microemulsion by two different kind of lipase, Novozyme 435 and Lipozyme IM were carried out (Figure 3). Lipozyme activity was found to increase as the incubation time was increased, with no difference at 48 and 72 hours. On the other hand, Novozyme activity was significantly higher than Lipozyme up to 6 hrs; the activity however decreased at 24 and 72 hrs. Gao *et al.*, (1999) showed that, with Novozyme, virtually no product was detected after incubating this enzyme with 2-bromomyristic acid and a trimeric sugar for 4 days, while Lipozyme catalysed the formation of the trimeric sugar ester at preparatively useful rates. Hernandez-Martin and Otero (2008) also found that alcoholysis of vegetable oils to produce biodiesel was faster with Lipozyme than with Novozyme. Large molar excess of the second substrate (alcohol or sugar) to fatty-acid residues was required by Novozyme (at least a 2:1 ratio) compared with Lipozyme which required only 0.33 molar ratio of substrates. The increase of some enzyme activity in the presence of surfactants can also occur as a result of surfactant-product or surfactant-substrate interactions.

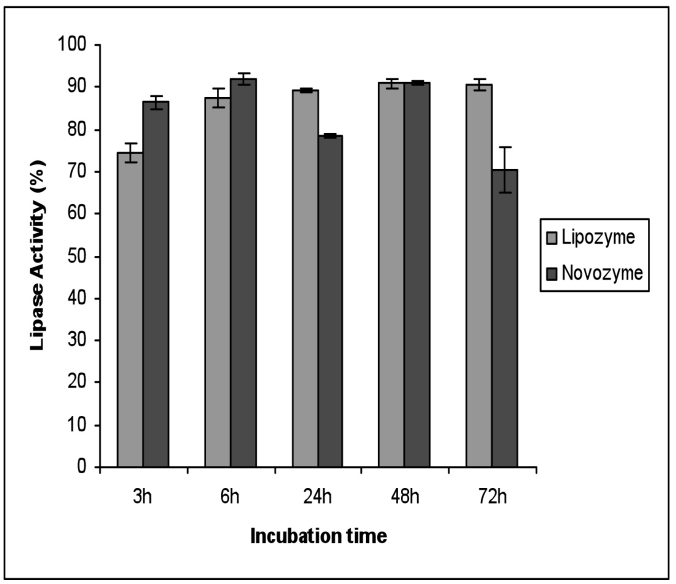

Figure 3: Effect of incubation time on lipase-catalysed synthesis of sugar ester in HTAB/Hexane-2M2B/Buffer microemulsion.

Figure 4 shows the result of lipase activity on sugar ester synthesis at different surfactant ratios. The ratios of 5/95 and 20/80 of HTAB resulted in more than 70% lipase activity compared to the other surfactant ratios. However, between the two, 5% water in 5/95 surfactant system produced higher yield compared to 15% water in 20/80 surfactant system. When performing these enzymatic reactions in organic solvent, the water produced has a distinct influence on the reaction equilibrium (Stamatis *et al.*, 1999) and may cause an inhibition of ester synthesis as well as damage to the enzyme molecule. Overall, it can be seen that the highest activity corresponds to a microemulsion with 5% of water concentration except for 20/80 system. Above the value, a decreasing enzyme activity was observed, which means that the addition of water has some influence on the enzyme activity. These is a good example on the advantage of microemulsion system since the water produced could be incorporated into the enzyme containing micro-droplets and consequently not affect the reaction equilibrium on the enzyme molecules

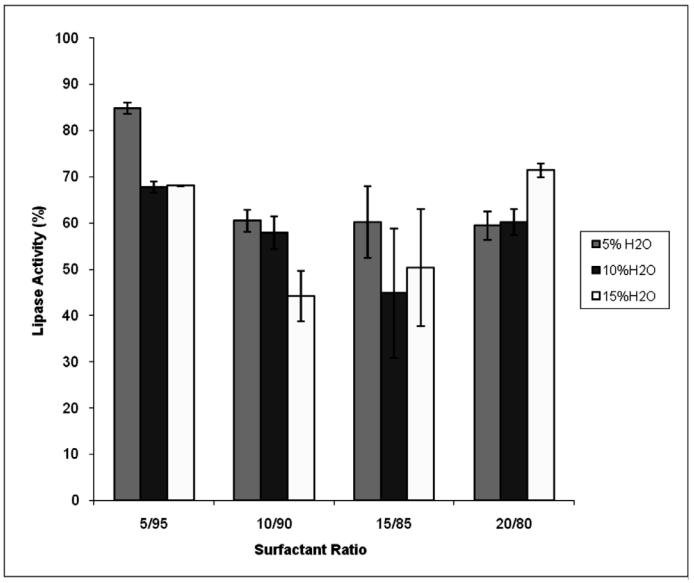

Figure 4: Effect of surfactant ratio and water concentration synthesis of sugar ester by Lipozyme in microemulsion.

Conclusion

Microemulsion offers a few advantages for the esterification of sugar ester in HTAB/Hexane-2M2B/Buffer systems. The requirements for bioconversion of hydrophobic substrates are fulfilled by using w/o-microemulsion as reaction media. The activity was highly dependent on the composition of the w/o-microemulsion used as media-reaction medium, mainly the water concentration. This w/o-microemulsion can therefore be proposed as a highly-potential reaction medium for high productions of the industrially-important sugar ester.

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