Biosynthesis of aroma esters in miniemulsion as potential green media

Fonseca L.P.[1•#], de Barros D. P. C.[1], Cabral J. M.S.[1], Weiss C. K.[2], Landfester K.[2]

[1]Institute for Biotechnology and Bioengineering, Lisbon, Portugal
[2]Max-Planck-Institute for Polymer Research, Mainz, Germany

[*] Luís Fonseca [#]luis.fonseca@ist.utl.pt

INTRODUCTION

The increasing sensitivity today for environmentally friendly process and preference for *green* technology and *natural* product favored the synthesis of flavor compounds via biotechnology route (Schrader J. et al., 2004).The goal of this study is to create an efficient enzymatic process for the production of aroma ester in a miniemulsion system. Miniemulsions are heterophase system where small droplets with high stability in a continuous phase are created by using intensive high shear force (in particular ultrasound) is called a miniemulsion. Here droplets with hydrophobic character can be obtained in an aqueous medium that represents the continuous phase. For a typical oil-in-water miniemulsion as system used in an esterification reaction an oil phase (as substrates), a hydrophobic agent (e.g. hexadecane), an emulsifier (usually nonionic surfactant), and water are homogenised by high shear (e.g. ultrasonification) to obtain homogenous and monodisperse droplets in the size range of 30 nm to 500 nm (Landfester et al., 2006). Esterification was performed by the addition of an enzyme as catalyst dissolved in the aqueous phase
A miniemulsions system shows a innovative potential as a system for the synthesis of alkyl esters.
The esterification reaction was performed with three different enzymes, lyophilized *Fusarium solani pisi* cutinase produced by S*acharomyces cerevisiae* SU50; commercial Lipase type VII from *Candida rugosa*, and Amano lipase PS from *Burkholderia cepacia*. The effect of the chain length of the alcohol and of the acids and influence of substrates concentration were investigated. A miniemulsions system shows a excellent potential as a system for the synthesis of flavour esters (e.g. for hexyl octanoate 96.2% with Amano lipase PS) and better stability for higher substrates concentration comparing with organic media system.

MATERIAL AND METHODS

Commercial lipase type VII from *Candida rugosa* (SIGMA) (1.150 U/mg against olive oil as substrate), Amano lipase PS from *Pseudomonas cepacia* (ALDRICH) (30,000 U/g against olive oil as substrate) and cutinase *Fusarium solani pisi* (Calado et al.,2003), (170 U/mg against p-nitrophenyl butyrate as substrate) were used for enzymatic esterification.
Reagents including hexanoic, heptanoic, octanoic (99%, SIGMA, Germany), decanoic and oleic acid (FLUKA,Germany) and ethanol abs. and hexanol (Merck, Germany) were used for esters synthesis while hexadecane 99% (SIGMA,Germany) and Lutensol® AT-50 (donation, BASF, Germany) were used to obtain miniemulsions. NaOH (Merck, Germany) as reagent and tetrahydrofuran (THF) (Merck, Germany) as solvent for titration were used. Paranitrophenyl – acetate, - butyrate, - valerate, - octanoate and palmitate (SIGMA, Germany) used evaluation of different enzymes in the hydrolysis reactions efficiency were of analytical grade. All other chemicals used were of analytical grade.

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CHARACTERIZATION OF THE CUTINASE PREPARATION

The protein concentration was measured by modified Lowry method using the Folin phenol reagent (Lowry et al., 1951).
The cutinase's estereolytic activity was assayed using a spectrophotometric method unless otherwise stated (Almeida C. F. et al., 2004) based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to nitrophenol (p-NP), a yellow compound easily identifiable and quantifiable by the absorbance at 400 nm. Specific activity of enzyme preparations were calculated as the ratio between estereolytic activity and protein concentration.

ENZYMATIC ESTERIFICATION REACTION IN MINIEMULSION SYSTEM

Unless otherwise stated a typical esterification reaction was carried out in 30 ml capped flasks used as a reactor with 20 ml of working volume with equimolar concentration of the substrates (acid and alcohol) (Table 1). Alcohol, acid, hexadecane, water and Lutensol AT50, are homogenized by magnetic stirring for 1h. The two phase system was ultrasonicated for 120 s with pulses of 5 s and pauses of 10s at 70 % amplitude (Branson sonifier W450 digital, tip size 6.5 mm) with ice cooling. Twenty ml of the miniemulsion was added into the reaction vessel containing the appropriate amount of enzyme (5 mg/ml of reaction mixture) (Fig 1).

Fig. 1 Principle of esterification in a miniemulsion system. Esterification was performed in a thermostated incubator (Advanced ChemTec PLS 4x4, 400 rpm) at 40°C unless otherwise stated.

This setup enabled parallel experiments by simultaneous use of 5 reactors against at least one blank without enzyme. Samples were withdrawn periodically (20 µl) using a needle and then mixed with NMR solvent (DMSO-d_6) (5ml) prior to NMR analysis.

	80% water content		50% water content		70% water content	
Acids	Ethyl esters	Hexyl esters	Ethyl est	Hexyl ester	Ethyl esters	Hexyl esters
	(M)	(M)	(M)	(M)	(M)	(M)
Hexanoic	1.0	0.75	3.0	2.25	1.7	1.2
Heptanoic	0.9	0.7	2.7	2.1	/	/
Octanoic	0.8	0.65	2.4	1.95	1.4	1.1
Decanoic	0.7	0.6	2.1	1.8	/	/
Oleic	0.5	0.4	1.5	4.5	/	/

Table 1 In the synthesis of ethyl- and hexyl- esters were used equimolar concentration of substrates in miniemulsion system.

The esterification reaction was performed in two different reaction operation modes: batch and discontinuous stepwise addition of alcohol (fed-batch). In the fed-batch operation mode the reaction was started with 25% of final substrate concentration and the additions of alcohol (25% of final alcohol concentration) were done every 15 min during the first hour of reaction.

RESULTS AND DISCUSSION

The ability of the lipase C*andida rugosa* type VII and *Fusarium solani pisi*. cutinase to catalyze the synthesis of ethyl- and hexyl esters was compared with the esterification activity of Amano lipase PS in the miniemulsion system.

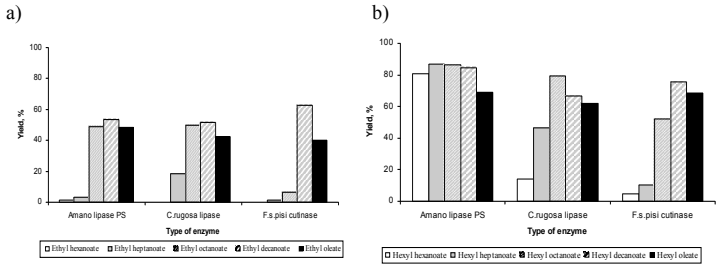

Fig. 2 Esterification yield for the synthesis of a) ethyl- and b)hexyl esters with three different enzymes

Similar to the Amano lipase PS catalyzed reactions, the esterification yield (24 h) was lower for ethyl esters than for hexyl esters (see Fig 6) .For ethyl esters the highest esterification yield was obtain for ethyl decanoate for all three enzymes: 53.5%, 51.5% and 62.5% respectively for Amano lipase PS, lipase *Candida rugosa* and cutinase *F.s. pisi*. Lipase *C. rugosa* show slightly higher preference for synthesis of ethyl heptanoate than the other two enzymes (Fig 2 a and b).
Amano lipase PS catalyzed reactions show higer conversions than the reactions catalyzed by the other two enzymes. Again it was found that with increasing chain length of the acid the yield of hexyl esters increases. Using C*. rugosa* lipase the maximum yield was obtained for hexyl octanoate

(79.4%), and for hexyl decanoate (66.7%) and hexyl oleate (62.1%). A similar reaction profile was observed with cutinase *F.s.pisi* showing the decrease of yield from hexyl decanoate (75.7%) to hexyl oleate (68.5 %).

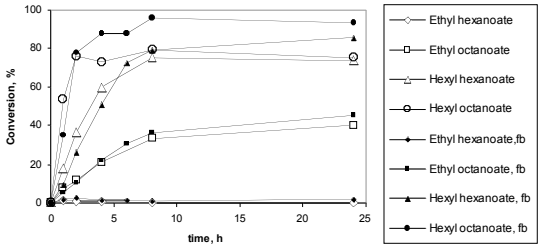

Fig. 3 Time courses of esterification under batch and fed-batch (fb) operational mode.

Results Fig. 3 suggest that consecutive alcohol supply during the enzymatic production of alkyl esters could be possible to perform with significant improvement on ester yield, especially in the case of hexyl esters. The enhancement of conversion for hexyl octanoate was 18.3 % if fed batch operational mode was applied and even for synthesis of ethyl octanoate an improvement (5.5 %) was observed. It shows that with fed batch mode it is possible to work more efficiently with hydrophilic or relatively hydrophilic substrates, such as ethanol and octanoic acid, in miniemulsion.

CONCLUSIONS

Amano lipase PS from *Burkholderia cepacia* showed good selectivity for the synthesis of hexyl esters. Cutinase show selectivity for longer chain of acid. Longer chain of acids and alcohol stabilize miniemulsion and provide better conversion to ester.
Fed-batch operational mode with consecutive supply of alcohol showed as a good strategy to improve esterification yield.

REFERENCES

• Schrader J. et al. (2004) Applied biocatalysis for the synthesis of natural flavour compounds-current industrial processes and future prospects. Biotechnology Letters 26 463-472.
• Landfester K (2006), *Synthesis of colloidal particles in miniemulsions*, Annu. Rev. Mater. Res., 36 231–279.
• Calado C. R. C. et al., (2003) *Development of a Fed-Batch Cultivation strategy for the Enhanced Production and secretion of Cutinase by a Recombinant Saccharomyces cerevisiae SU50 Strain* Journal of Bioscience and Bioengineering 96 141-148.
•Lowry, O. H et al. (1951). *Protein measurement with the Folin-Phenol reagents*. J. Biol. Chem. 193 265-275.
• Almeida C. F. et al. (2004) *Flow injection analysis system for on-line cutinase activity assay*, Analytica Chimica Acta 502 115-124.