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Journal of Molecular Catalysis B: Enzymatic 29 (2004) 155–161
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Sugar ester synthesis by a mycelium-bound *Mucor circinelloides* lipase in a micro-reactor equipped with water activity sensor

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Received 3 July 2003; received in revised form 29 January 2004; accepted 2 February 2004

Available online 20 April 2004

Abstract

The mycelium-bound *Mucor circinelloides* lipase was used for the synthesis of esters of saccharides and fatty acids in 37 ml reactor equipped with magnetic stirrer and water activity sensor. Either di-*n*-pentyl ether or the mixture of di-*n*-pentyl and petroleum ethers were applied as reaction media. Water activity sensor provided *on-line* monitoring of this parameter and control of continuous processes of ester synthesis. It was found that two natural antioxidants, i.e. carotene and astaxanthin activated this lipase in organic solvents that could be beneficial for the synthesis of esters of compounds sensitive to oxidation, e.g. polyunsaturated fatty acids.

Keywords: Mucor; Mycelium-bound lipase; Saccharides esters; Water activity; Activation

1. Introduction

Esters of saccharides and fatty acids are natural, “green”, non-ionic surfactants. Their HLB (hydrophilic–lipophilic balance) varies from 1 to 18. These compounds are synthesized from natural components of foods and show prebiotic properties, and therefore they are applied for food production as dietetic fats, emulsifiers, plasticizers, and as antimicrobial and protective coatings for fruit. Sugar esters have attracted attention of biotechnologists because they consist of two inexpensive, renewable and easily available raw materials such as sugar and fat/oil [1–5].

Large-scale production of the majority of sugar esters has mainly employed chemical synthesis. Application of enzymes, in particular lipases, as biocatalysts of these reactions is an alternative, “green” way to be used instead of chemical technologies. Bioconversions catalyzed by lipases were shown to be superior to chemical processes [6]. In organic media lipases catalyze ester bond formation and produce sugar esters [6–12]. This reaction requires low water activity of the medium, favorable for displacement of the reaction equilibrium towards synthesis reaction [13,14]. However, the by-product of this reaction is water, which disrupts ester bond synthesis, and must be discarded to avoid reduced productivity of esters.

Amounts of water added to the reaction mixture and released as a by-product of the synthesis reaction affect the activity of enzymes in organic media [15–19] and affects this process yield. Water content in reaction medium can be expressed either as its concentration or as water activity (a_w). Water activity describes the continuum of energy states of water molecules present in a system. Water molecules in a sample are linked via dipole–dipole forces, ionic bonds (H_3O^+ or OH^-), van der Waals forces and hydrogen bonds [20]. These interactions involve either exclusively water molecules or water molecules and other polar atoms present in the reaction mixture. The thermodynamic water activity (a_w) is probably the best parameter to characterize the hydration of the system [13–22].

Wehtje et al. [7] measured changes in water activity throughout ester synthesis, catalyzed by lipases in organic solvent-containing media. They used a system composed of a semi-permeable silicone tubing and saturated salt solutions. The system was suitable for different reactors (tank reactor, continuous stirred-tank reactor (CSTR), and tubular reactor). Their studies also demonstrated that the sensor,

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doi:10.1016/j.molcatb.2004.02.009

which measured water activity of organic media based on determination of water partial pressure in gaseous phase above the solvent, showed a_w values with a delay, and therefore it was not appropriate for continuous a_w monitoring.

Our studies focused on sugar ester synthesis catalyzed by the intracellular *M. circinelloides* lipase in a mixture of di-*n*-pentyl and petroleum ethers and in the presence of astaxanthin or carotene, which dependently on their concentration in reaction mixtures activated or inhibited this lipase. Reactions were carried out in a jar reactor equipped with magnetic stirrer and with the water activity digital sensor Aw VC-DIO (Rotronik), which continuously measured a_w of the reaction medium.

2. Materials and methods

2.1. Materials

M. circinelloides strain was obtained from the pure culture collection of the Institute of Technical Biochemistry of Technical University of Lodz. Caprylic acid (a_w^0 of 0.20), oleic acid (a_w^0 of 0.16), linoleic acid (a_w^0 of 0.10), sucrose (a_w^0 of 0.13), glucose (a_w^0 of 0.08) and *trans*-β-carotene (all were analytical grade) were purchased from Sigma, Baker, Fluka and ICN. Astaxanthin in a form of mono-, di- or triesters was extracted from shrimp carapaces as described in [23]. Regulating substances (also termed ambivalent factors) were extracted from *M. racemosus* mycelium as reported in [23]. Symbol a_w^0 denotes an initial water activity of reaction mixture components.

2.2. Preparations of lipases

The mycelium-bound *M. circinelloides* lipase preparations were obtained as was described elsewhere [23–25]. After drying, the immobilized in situ lipase was ground in a vibrating mill to particles 3.5–5.0 μm in diameter (a_w^0 of 0.48).

2.3. Synthesis of esters in micro-reactor

Synthesis of esters was carried out at 50 °C in a jar reactor (diameter of 40 mm, height of 40 mm, capacity of 37 ml) equipped with a magnetic stirrer (120 rpm). Reaction mixtures contained: 2 mmol of sugar, 2 mmol of fatty acid and 0.2 g of the lipase suspended in 10 ml of organic solvents (di-*n*-pentyl ether, or the mixture of di-*n*-pentyl and petroleum ethers), enriched with astaxanthin, carotene or regulating substances, derived from *M. racemosus*. The reagents were preincubated in the reactor for approximately 30 min, before adding the lipase. Water activity, changes in relative humidity (RH) and temperature of reaction medium were monitored throughout ester synthesis using the water activity digital sensor Aw VC-DIO (Fig. 1) coupled with instrument HW 2 (Rotronik) and computer. The sensor was

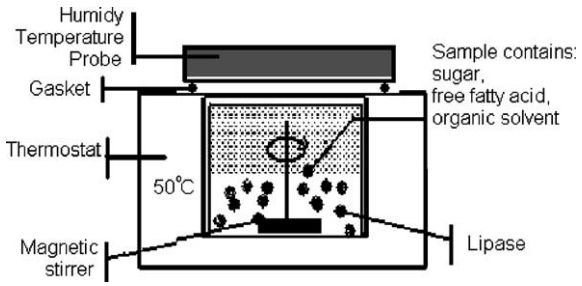

Fig. 1. Schematic representation of enzymatic reactor equipped with an *on-line* relative humidity sensor.

calibrated with three standards (a_w of 0.10, 0.35 and 0.80) successively placed in the empty reactor.

Di-*n*-pentyl ether was saturated with water as follows: 200 ml of ether and 50 ml of water were incubated at 50 °C on a rotary shaker (120 rpm) for 5 days. Water dissolved in the solvent was assayed according to Karl Fischer by using the instrument produced by Mattler (model DL18).

Reaction conditions different from that described above are presented under the figures.

2.4. Synthesis yield

Free fatty acids were titrated with 0.05 M NaOH up to pH 10.0 using TitroLine (Schott) titrator. The synthesis efficiency (mol%) was calculated from the amount of acid consumed in the reaction. Esters concentration was determined by using Beckman Gold HPLC system (μ-Spherogel 50 Å column; 300 mm × 7.7 mm, elution with tetrahydrofurane; refractometric detector).

3. Results and discussion

3.1. The effect of initial water activity on reaction of saccharide ester synthesis

Structure and physicochemical properties of solvents (e.g. log *P* and dielectric constant) and water activity in reaction medium are the most important parameters affecting enzyme activity and stability [7,26–29].

Earlier studies on sugar ester synthesis by *M. circinelloides* lipase revealed that di-*n*-pentyl ether (log *P* of 3.9 [26]) was the best reaction medium for reactions catalyzed by this enzyme [23,25,30]. Recently, novel reaction media composed of solvents structurally similar to di-*n*-pentyl ether have been tested. Yields of reactions carried out in di-*n*-butyl ether, *tert*-butyl alcohol, *tert*-pentyl alcohol, isopropyl ether and ethyl ether were very small in contrast to that in di-*n*-pentyl ether, in which glucose caprylate synthesis yield was 69% (Table 1).

The lipase, glucose and sucrose are virtually insoluble in di-*n*-pentyl ether [30], and therefore these three substances form the solid phase of reaction mixture, which

Table 1
Glucose caprylate synthesis yields in various solvents

Solvent	log *P*	a_w^0 (50 °C)	Yield (%)
Di-*n*-pentyl ether	3.90	0.26[a]	69.2
Petroleum ether	3.20	0.08	4.1
Di-*n*-butyl ether	3.08	0.09	7.2
Tert-butyl alcohol	0.80	0.08	6.0
Tert-pentyl alcohol	1.30	0.13	6.6
Isopropyl ether	1.90	0.08	0
Ethyl ether	0.85	0.08	0

Reaction time 2 h.
[a] Commercial di-*n*-pentyl ether.

is the heterogeneous system. To enhance the solubility of saccharides, either DMSO or 2-pyrrolidone were added. The effect of DMSO on enzymatic synthesis of sugar esters was reported elsewhere [31–33]. Both these co-solvents decreased the activity of *M. circinelloides* lipase. The 2% DMSO in di-*n*-pentyl ether (a_w^0 of 0.07) halved productivity of ester synthesis. The increase in synthesis yield (a rise of approximately 6%) was achieved by grinding of the saccharide particles and a decrease in an initial water activity (a_w^0) of the substrates of this reaction (P_2O_5, molecular sieve 4 Å). The solubility of saccharides in di-*n*-pentyl ether was slightly increased by saturation of this solvent with water. However, this operation reduced productivity of ester synthesis (a drop of 10%), carried out in the stirred reactor.

Stirring significantly affects reaction yield in heterogeneous systems. For instance, when the synthesis of sucrose caprylate, catalyzed by *M. circinelloides* lipase, was carried out on a reciprocal shaker (240 rpm) in di-*n*-pentyl ether saturated with water (water concentration of 4.37 mg ml^{-1} and a_w^0 of 0.45), the conversion of caprylic acid was 76% after 2 h, whereas in the anhydrous di-*n*-pentyl ether (water concentration of 0.34 mg ml^{-1} and a_w^0 of 0.07) the yield of ester synthesis was only approximately 30% [23]. In our recent experiments carried out in a stirred micro-reactor (120 rpm), higher sucrose caprylate synthesis yield was observed in anhydrous di-*n*-pentyl ether. After 2 h of the reaction conducted at 50 °C in the solvent saturated with water (a_w^0 of 0.45), the ester synthesis yield was 75% (Figs. 2 and 3), and in the solvent dehydrated with the molecular sieve (a_w^0 of 0.07), the synthesis efficiency reached 82% (Fig. 2). In commercial di-*n*-pentyl ether with a_w^0 of 0.26, the

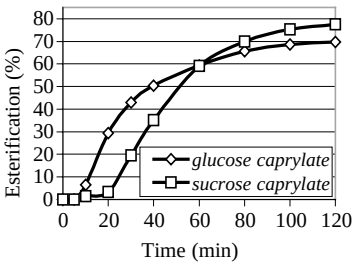

Fig. 2. The dynamics of accumulation of glucose and sucrose caprylate in di-*n*-pentyl ether (a_w^0 of 0.26 at 50 °C).

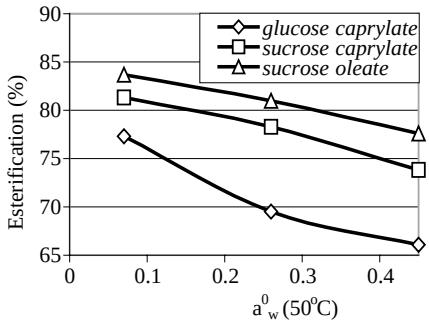

Fig. 3. The effect of initial water activity of di-*n*-pentyl ether on glucose and sucrose ester synthesis yields; reaction time 2 h.

yield of sucrose caprylate synthesis was in-between these values (78%, Fig. 3). The correlation between the solvent's a_w^0 and synthesis yields of other esters is shown in Fig. 3. Stirring of the reaction mixture improved contact of solid saccharide particles and immobilized lipase, and markedly enhanced ester synthesis efficiency in anhydrous solvent. Earlier studies revealed that the mycelium-associated *M. circinelloides* lipase catalyzed the hexadecanolide synthesis from solid 16-hydroxyhexadecanoic acid, suspended in the reaction mixture [24]. Higher concentration of this acid in reaction mixture resulted in enhanced lactone production.

3.2. Changes in relative humidity of reaction mixture during sugar ester synthesis

Studies on the effect of water activity on glucose and sucrose ester synthesis by *M. circinelloides* lipase in organic media demonstrated that this enzyme was active both in virtually anhydrous di-*n*-pentyl ether (a_w^0 of 0.07) and in the solvent saturated with water (a_w^0 of 0.45) (Fig. 3).

Water is a by-product of ester synthesis, and if it is not expelled from the medium (e.g. by absorption or distillation under vacuum [7]) its amount rises gradually. Changes in water content in the reaction medium throughout ester synthesis, conducted in enzymatic reactor were monitored by using the *on-line* sensor. The measurements were delayed that resulted from the technical characteristics of the sensor. According to our observations, the delay was approximately 5 min at the beginning of the measurements. The low accuracy of this determination was caused by water released in the reaction. It disturbed the state of equilibrium of water distribution between two phases because their volume ratio was not constant, and therefore the sensor showed the relative humidity and not the water activity of the system.

The correlation of these two parameters is given by the following equation:

$$a_w = \frac{p}{p_0} = \frac{\mathrm{ERH}\,(\%)}{100}$$

where p is the vapor pressure of water in a material, p_0 the vapor pressure of pure water, ERH the relative humidity in equilibrium state and ERH/100 the same as a_w in the state of

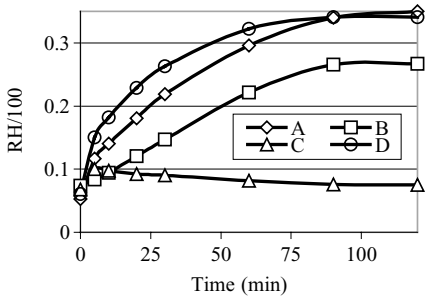

Fig. 4. The effect of glucose caprylate synthesis conditions on changes in relative humidity of the reaction medium. Reaction was carried out in di-*n*-pentyl ether dried with molecular sieve 4 Å prior to use (a_w^0 of 0.07 at 50 °C). Lipase preparation was dried with acetone (A, C, D) or with P_2O_5 (B). In experiments C and D, reaction mixtures additionally contained molecular sieve or silica gel, respectively. The maximum synthesis yields for A, B, C and D were 75, 71, 88 and 77%, respectively.

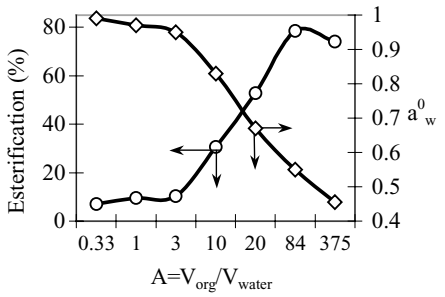

Fig. 5. The correlation between phase volume ratio (*A*) of the biphasic system (di-*n*-pentyl ether/water), its a_w^0 at 50 °C, and sucrose caprylate synthesis yield in this medium; reaction time 20 h.

equilibrium, when water distribution in all phases remains constant.

Figs. 4 and 7 present dynamics of an increase in water content throughout enzymatic ester synthesis expressed as changes in RH/100.

In the reactor equipped with the a_w monitoring sensor and a magnetic stirrer, the highest yield of glucose caprylate was achieved in anhydrous di-*n*-pentyl ether (a_w^0 of 0.07), and in the presence of a water-absorbing molecular sieve 4 Å. The presence of molecular sieve in this system provided the constant, low value of RH/100, beneficial for the reaction of glucose and sucrose ester synthesis (Fig. 4C). Water molecules produced in the reaction increased RH/100 in the reaction mixture which contained di-*n*-pentyl ether dehydrated prior to reaction but did not contain the molecular sieve (Fig. 4B and D). Dehydrating of the lipase preparation with P_2O_5, prior to its application for ester synthesis, resulted in a decrease in the yield of this process from 75 to 71%, as compared to that catalyzed by lipase, which was not treated with P_2O_5 (Fig. 4B and A). The preparation of mycelium-anchored *M. circinelloides* lipase adsorbed a part of water liberated during the reaction, thus decreasing RH/100 in the system.

The efficient ester synthesis requires large concentrations of reagents. Water formed as a by-product of synthesis of 2–3 mol ester/l and not discarded from the reaction mixture, generates the biphasic system: organic solvent/water, with the phase volume ratio (*A*) of 20–40 ($A = V_{org}/V_{water}$, where V_{org} is the organic phase volume in the reaction mixture and V_{water} the water phase volume at the end of the reaction). Our earlier studies demonstrated that the index *A* and water volume of the biphasic system affected ester synthesis productivity achieved in the post-stationary state. For sucrose caprylate synthesis, the maximum of the function $K_0 = f(A)$ (where K_0 is the equilibrium constant of reaction in organic phase) corresponds to *A* in the range 40–80 (dependent on fatty acid applied for the synthesis). An increase in the water phase volume of the biphasic system decreases synthesis productivity [30].

Water activity of model biphasic system depends on *A* (Fig. 5). A rise in water phase volume of biphasic system di-*n*-pentyl ether/water (a drop in *A*) increases water activity in this system. The highest yield of sucrose caprylate synthesis was found for *A* of 84, which corresponded to a_w of 0.55. Further increase in the volume of water phase in this system augmented a_w and reduced sucrose caprylate synthesis yield (Fig. 5). To avoid a direct contact of water droplets with the sensor, which may have a negative impact on accuracy of a_w measurements, the experiments were carried out in a reactor without agitation. Due to the difference in densities, water was the bottom phase of the biphasic system.

In our experiments, the substrate concentrations were 0.2 mol l^{-1}. Because the experiment was carried out in anhydrous solvent, the maximum amount of water, which could be produced if 100% conversion of the reagents were achieved, was 36 mg, and it was insufficient to saturate di-*n*-pentyl ether in the reactor (40 mg). Measurements of a_w throughout ester synthesis carried out in the solvent with the initial a_w^0 of 0.07 showed that after 2 h di-*n*-pentyl ether was not saturated with water produced in the reaction, because RH/100 of the medium was only 0.35, thus below that corresponding to the state of saturation at 50 °C (Fig. 4). Therefore, we think that the reaction occurred in the monophasic system. From theoretical point of view, the term “monophasic” is rather conventional with reference to the system composed of two different solvents. Each enzyme-catalyzed synthesis in organic solvents occurs in a biphasic system: organic solvent/water (if we take into consideration the closest microenvironment of enzyme) because the essential water layer constitutes the water phase, which is always present in the reaction mixture. Therefore, one may ask if the essential water layer should be considered as the second liquid phase of the biphasic system.

It was found earlier that the initial water activity strongly affected the productivity of fructose palmitate synthesis, catalyzed by immobilized *Candida antarctica* lipase in 2-methyl-2-butanol [13]. The lowest water activity (0.07) resulted in the highest synthesis yield, however, the enzyme specificity was changed under these conditions. Low water content in reaction mixture resulted in its high hydrophobicity and reduced fructose solubility. Therefore,

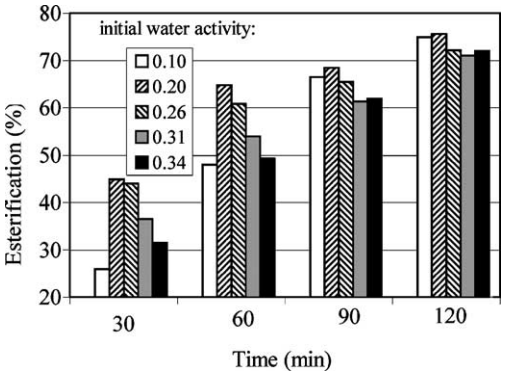

Fig. 6. The effect of initial water activity at 50 °C of di-*n*-pentyl ether on glucose caprylate synthesis yield.

monopalmitate was a preferred substrate for further esterification. Molecules of lipase, both reagents and di-*n*-pentyl ether contain polar atoms, which attract water present in reaction medium [20], and that influences the rate of synthesis. Competition between water and fructose affected the initial synthesis velocity [13]. Smaller reaction rates in the media with higher value of a_w, presumably resulted from the limited contact of palmitic acid and lipase, which was surrounded by water layer. As revealed by electron microscopy, layers of water molecules attracted by lipase molecules protected the enzyme from its lipophilic substrate, and gave rise to aggregation of enzyme [34].

In our studies 10, 20, 30, 40 and 50 μl aliquots of water were added to 10 ml portions of anhydrous di-*n*-pentyl ether. Water activity of these mixtures was measured after 24 h incubation at 50 °C. Values of a_w^0 were: 0.1, 0.20, 0.26, 0.31, and 0.34, respectively. All these solvents were used as media for glucose caprylate synthesis. After 30 and 60 min of the reaction, the product yields were dependent on the initial a_w^0 of the solvent, with maximum for a_w of 0.20 (Fig. 6). However, after 2 h of the reaction these yields were almost the same and ranged from 70 to 75% (Fig. 6). Water produced in this reaction caused a continuous increase in RH/100 measured *on-line* in the reaction mixture. Changes in RH/100 are presented in Fig. 7. After 2 h of synthesis, carried out in solvents with a_w^0 of 0.31 and 0.34, the values of RH/100 were larger than a_w^0 of di-*n*-pentyl ether saturated with water (at 50 °C), that indicated generation of biphasic system.

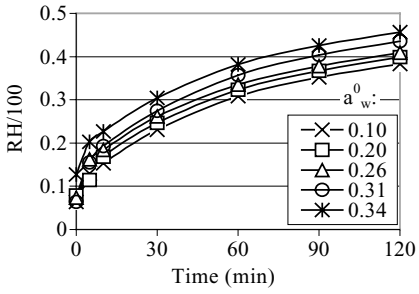

Fig. 7. Changes in relative humidity during glucose caprylate synthesis in di-*n*-pentyl ether with different initial water activity (a_w^0 at 50 °C).

3.3. Lipase activation in organic solvents

It is difficult to explain why some solvents are better media for enzyme-catalyzed processes than the other. Proteins usually individually optimize their three dimensional structure and interactions with solvents [13]. Generally, apolar organic solvents, unable to form multiple hydrogen bonds, generate conformational changes, which result in stronger electrostatic bonds inside protein molecule and stiffen its edifice. This constrains conformational changes of the enzyme upon catalysis and reduces its activity [29,35].

The coat of water molecules and the structure of active site stabilize the active conformation of lipases in apolar solvents. Because the catalytic pocket of these enzymes is hydrophobic, it can be protected by substances with the same character [6,29].

Antczak et al. [24] noticed that the highest productivity of synthesis of 16-hydroxyhexadecanoic acid lactone, catalyzed by the *M. circinelloides* lipase, was achieved in the mixture of toluene and petroleum ether, with log *P* of 2.93. The mixture of di-*n*-pentyl and petroleum ethers was applied for synthesis of sugar esters. Petroleum ether was found to be a very good solvent for synthesis of esters of higher fatty acids and aliphatic alcohols [36]. It also protected the *M. circinelloides* lipase activity under extreme conditions (1 h, 100 °C) [36].

In studies on optimization of medium composition for ester synthesis reaction the mixtures of di-*n*-pentyl ether and the aforementioned solvents were applied. Higher yields of sucrose and glucose ester synthesis were achieved in a mixture of di-*n*-pentyl and petroleum ethers, as compared to reactions carried out in di-*n*-pentyl ether. The highest productivities of sucrose caprylate, oleate and linoleate were observed in the mixture of organic solvents with log *P* of 3.62. In contrast, the best productivity of glucose caprylate synthesis was detected in the mixture with log *P* of 3.74 (Table 2).

Activation of lipase by *N*,*N*-dimethylformamide added to organic solvent was for the first time detected by Zaks and Russell [37], who thought that this compound modified water layer of the enzyme. Antczak et al. [24] proved that the presence of *N*,*N*-dimethylformamide or pyridine 10-fold increased the lipase activity in hexadecanolide synthesis. Apart from pyridine and *N*,*N*-dimethylformamide also other synthetic and natural substances regulated activity of *Mucor* lipases in organic solvents. They include piperazine, diethylamine (DEtA), triethylamine (TEtA), as well as some substances with the lipid character, extracted from *M. circinelloides* and *M. racemosus* mycelia (*regulating substances*). Dependent on concentration in reaction mixture they either activated or inactivated the *Mucor* lipase [23,25,36]. Our earlier studies on activation of the homogeneous *M. circinelloides* lipase, dissolved in toluene, revealed

Table 2
Yields of sucrose and glucose caprylate synthesis in di-*n*-pentyl and petroleum ether

Di-*n*-pentyl ether (ml)	Petroleum ether (ml)	log *P*	Glucose caprylate		Sucrose caprylate		Sucrose oleate		Sucrose linoleate	
			a_w^0 (50 °C)	Yield (%)	a_w^0 (50 °C)	Yield (%)	a_w^0 (50 °C)	Yield (%)	a_w^0 (50 °C)	Yield (%)
2.5	0	3.90	0.26	58.2	0.26	57.5	0.26	56.8	0.26	56.1
2.0	0.5	3.74	0.20	65.5	0.20	65.6	0.20	60.8	0.20	59.3
1.5	1.0	3.62	0.16	62.3	0.16	66.9	0.16	66.1	0.16	66.1
1.0	1.5	3.48	0.12	58.2	0.12	50.9	0.12	57.6	0.12	59.1
0.5	2.0	3.34	0.09	34.3	0.09	23.4	0.09	37.3	0.09	37.8
0	2.5	3.20	0.07	5.1	0.07	0	0.07	0	0.07	0

Reaction time 60 min.

that this enzyme was activated not only in aqueous media, but also in organic solvents. The molecular background of this phenomenon seems to be similar to the interfacial activation of lipases in water solutions (namely the "lid"-helix translocation), thought the reason is different [25].

Natural lipase activators, such as astaxanthin, carotene and *regulating substances* from *M. racemosus* were used in our latest studies. All of them display antioxidant properties. The presence of 4 mg ml^{-1} astaxanthin in the reaction medium increased the productivity of sucrose caprylate, oleate, and linoleate synthesis (Fig. 8A). β-Carotene had the similar beneficial impact on sugar ester synthesis yield (e.g. glucose and sucrose caprylate, 0.17 mg ml^{-1} of carotene, Fig. 8B) synthesis by the *M. circinelloides* lipase.

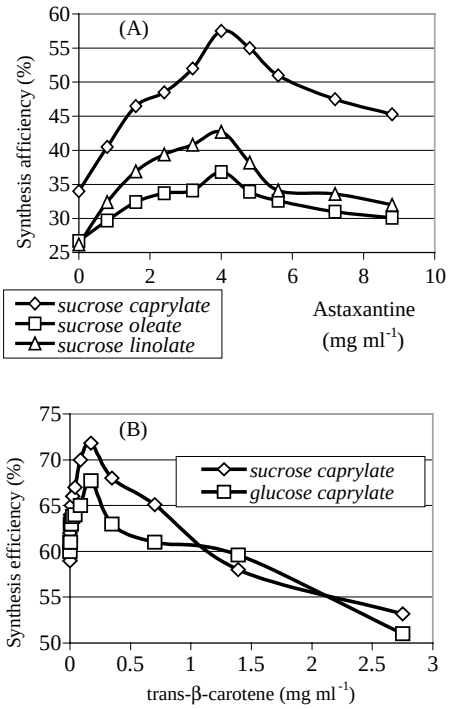

Fig. 8. An influence of selected substances on sugar ester synthesis yield in di-*n*-pentyl ether with an initial water activity of 0.07 at 50 °C. Reaction times: (A) sucrose caprylate, 20 min; sucrose oleate, 15 min; sucrose linolate, 15 min; (B) 60 min.

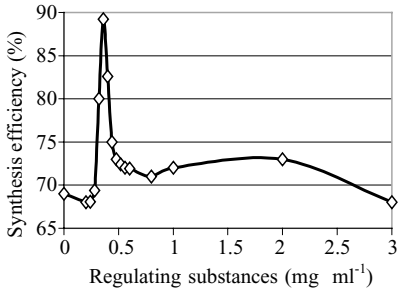

Fig. 9. An influence of regulating substances from *M. racemosus* on glucose caprylate production in di-*n*-pentyl ether with an initial water activity of 0.07 at 50 °C; reaction time 2 h.

The regulating substances derived from *M. racemosus* biomass are presumably derivatives of cholic acid or carotenoids. Almost 90% yield of caprylic acid esterification was achieved, when 0.36 mg ml^{-1} of regulating substances were present in the reaction mixture (Fig. 9).

4. Conclusions

The initial water activity of the organic solvent strongly affects the initial velocity of ester synthesis. Water released in this reaction increases water activity of reaction mixture, and its excess may lead to generating of the biphasic system, which seems to be beneficial for ester synthesis by the *M. circinelloides* lipase.

The applied method of water activity measurements facilitates on-line monitoring of this parameter. However, practical use of this method requires more sensitive sensor with faster response, designed for a_w measurements in organic solvents. Accurate determination of free water concentration in organic medium can facilitate generation of biphasic systems with optimum phase volume ratio (*A*) that is particularly useful in continuous synthesis processes.

Natural antioxidants, such as carotene and astaxanthin, and substances isolated from *Mucor* mycelium (*regulating substances*) can be applied as *M. circinelloides* lipase activators in synthesis of sugar esters in organic solvents. They can protect substrates susceptible to oxidation, such as polyunsaturated fatty acids.

Acknowledgements

The authors gratefully acknowledge the financial support of the State Committee for Scientific Research (KBN) for this work under grant No. PBZ KBN 021/PO6/99/22.

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