ISSN 1330-9862
(FTB-3028)

original scientific paper

Simple and Mixed Reverse Micelles as Potential Bioreactors for Enzymatic Synthesis of Alkyl Glycosides – Environmentally Friendly Surfactants

*Irina Mladenoska**

University Sts Cyril and Methodius, Faculty of Technology and Metallurgy, Department of Biotechnology and Food Technology, Rudjer Boskovic 16, MK-1000 Skopje, Republic of Macedonia

Received: February 10, 2012
Accepted: May 11, 2012

Summary

Two types of microemulsion reaction systems: simple and mixed reverse micelles have been investigated as potential bioreactors for a transglycosylation reaction catalyzed by three microbial β-galactosidases: fungal *Aspergillus oryzae*, yeast *Kluyveromyces marxianus* and bacterial *Escherichia coli* β-galactosidase. Several issues such as: the effect of the degree of hydration on the total enzyme activity, the enzyme selectivity towards glycon donors, the interphase quality of simple and mixed micelles and the effect of addition of nonionic cosurfactants on the enzyme activity were all discussed. Both *p*-nitrophenyl-β-D-galactoside and *p*-nitrophenyl-β-D-glucoside were used as activated substrates. The total activity of all three examined enzymes showed to be strongly dependent on the degree of hydration of reverse micellar systems and had the highest values at the degree of hydration close to the level of saturation. The highest activity that the *A. oryzae* β-galactosidase had in simple micelles per mass of protein was 1.8 mmol/(min·mg). The ratio of β-galactosidase and β-glucosidase activities had values above 1 for all the enzymes entrapped in simple micelles, which means that their selectivity towards the *p*-nitrophenyl-β-galactoside as a glycon donor was strict and higher than that towards the *p*-nitrophenyl-β-glucoside. This ratio for the *A. oryzae* β-galactosidase was 1.68. The addition of nonionic cosurfactants had a positive effect on the enzyme activity. Thus, the total activity of *A. oryzae* β-galactosidase in dioctyl sodium sulfosuccinate/polyethylene glycol (AOT/PEG) mixed micelles with a mass ratio ζ(AOT, PEG)=1 was 1.5 times higher and in AOT/Tween mixed micelles with a mass ratio ζ(AOT, Tween)=0.033 it was even more than 3.5 times higher than the activity of enzyme entrapped in simple micellar reactor. The latter activity was even 4.22 times higher than that of *A. oryzae* β-galactosidase used as a crude enzyme preparation.

Key words: reverse micelles, mixed reverse micelles, enzymatic synthesis, alkyl glycosides, surfactants

Introduction

Utilization of hydrolytic enzymes for synthetic reactions in unconventional reaction media is nowadays well developed area of bioorganic synthesis and a basis for clean technological processes (*1–6*). These processes, known also as greenchem processes, are characterized by mild reaction conditions, utilization of renewable resources and selective production of specialty biocompounds with minimal side products and minimal waste generation (*7*). Pollution prevention rather than cleaning of the already damaged environment is the paradigm of these clean technological processes.

*Corresponding author; Phone: ++389 2 3064 588/239; E-mail: irinaetf@t-home.mk

Enzymatic synthesis of alkyl-β-glycosides, biodegradable, biocompatible, eco-friendly nonionic surfactants, is certainly an example of this kind of processes and has attracted much attention in the last decade (*8–10*). Prepared from renewable resources, sugars and fatty alcohols, these unique surface-active compounds have broad potential application in pharmaceutical, food or detergent industries and are used for special medical and analytical purposes as well (*11*). Special and very efficient alkyl glycosides with very long carbohydrate chains, which are very difficult to be synthesized by classical chemical methods, are today successfully synthesized enzymatically (*12*).

Glycosidases are a group of hydrolases capable of catalyzing alkyl-β-glycoside synthesis *via* two reaction modes: a reversed hydrolysis and a transferase reaction, the transglycosylation (*13,14*). While in the reverse hydrolysis reaction the thermodynamic equilibrium sets the limit for the reaction yield, the transglycosylation is a kinetically controlled reaction and the yield depends on the reaction conditions and enzyme properties (*15,16*). In transferase reactions, activated substrates, such as *p*--nitrophenyl-β-glycosides, are usually used. An environment with low water content is necessary in order to avoid the secondary hydrolytic reactions, the hydrolysis of the already formed alkyl glycoside, so water-immiscible alcohols are used both as media and as substrates (*10*). Besides the water content, the miscibility between a very hydrophilic sugar and a very hydrophobic alcohol moiety might also be a problem. Therefore, the interfacial area between the hydrophilic and the hydrophobic moieties, as well as the enzyme dispersibility in the organic solvent are very important parameters that determine the process yield.

Preparation of enzymes for their use in organic solvents is often a necessity and is usually performed in order to lower the diffusional limitations, to enlarge the interfacial area between the immiscible substrates and to achieve better dispersibility of the enzyme in the media (*4,17*). For this purposes enzymes have been modified using various modification and immobilization techniques, such as an adsorption and deposition onto solid supports, modification with surfactants and preparation with different kinds of additives.

The system of reverse micelles can be used as a reaction system suitable for synthetic reactions in unconventional media (*18,19*). These microreactors have shown to be convenient for these types of reactions due to their low water content, amphiphilic characteristics and small size. Reverse micelles are formed in organic solvents where small droplets of water are surrounded by a surfactant monolayer. The enzyme is present in the water droplets, together with the hydrophilic substrates, while the continuous phase consists of an organic solvent in which the hydrophobic substrates are dissolved. In this way a very broad reaction interfacial area is achieved, approx. 100 m^2/mL of microemulsion (*20*).

Reverse micelles have so far been used for partial glyceride synthesis (*20,21*), alkyl-β-glycoside synthesis (*18*), galactooligosaccharide synthesis (*22*) and chitooligosaccharide synthesis (*19*). However, to the best of our knowledge, there are not many papers published on the transglycosylation activity of glycosidases in mixed reverse micelles.

In this paper the transglycosylation activity of three different microbial galactosidases was investigated: of the fungal *Aspergillus oryzae*, the yeast *Kluyveromyces marxianus* and the bacterial *Escherichia coli* β-galactosidase. The enzymes were used in two different forms: freely dispersed in the organic medium and entrapped in simple and mixed reverse micelles. The effect of the degree of hydration (*r*(water, surfactant) value) on the enzyme activity and selectivity towards the glycon donors was also examined. The *p*-nitrophenyl-β-glucoside and *p*-nitrophenyl-β-galactoside were both used as activated substrates.

Materials and Methods

Materials

Three microbial β-galactosidases were used: the first one from *Aspergillus oryzae* (≥8.0 U per mg of solid), the second from *Kluyveromyces marxianus* (≥2600 U per g of solid), and the third enzyme was from *Escherichia coli* (≥500 U per mg of protein). All of them were purchased from Sigma Chemicals (St. Louis, MO, USA). Organic solvents, hexanol and isooctane were received from Sigma Chemicals, while acetonitrile was a Merck (Darmstadt, Germany) product. Other chemicals, such as sugar substrates, *p*-nitrophenyl-β-D-galactoside and *p*-nitrophenyl-β-D-glucoside, as well as hexyl-β-D-galactoside and molecular sieves (UOP TYP 3A) for drying solvents (0.3 nm diameter) were also purchased from Sigma Chemicals. AOT (dioctyl sodium sulfosuccinate) was also a product of Sigma Chemicals, as well as the monomethoxypolyethylene glycol (PEG, M_r=4000) and polyoxyethylene sorbitan trioleate (Tween 85).

Methods

Enzymatic reactions

p-Nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-glucoside were dissolved in hexanol (dried with 0.3-nm molecule sieves) to a concentration of 10 mM. Different volumes (40–280 μL) of aqueous buffer containing the desired amount of enzyme were added. The buffers used were: 50 mM citrate buffer, pH=5.0, for *A. oryzae* β-galactosidase, or 50 mM phosphate buffer, pH=7.0, containing 1.0 mM $MgCl_2$ for *E. coli* and *K. marxianus* β-galactosidases. The enzyme-buffer solutions had the following concentrations (in g/L): for *A. oryzae* β-galactosidase 0.4, for *K. marxianus* β-galactosidase 0.25 and for *E. coli* β-galactosidase 0.05. Volumes of 40 μL of thus prepared solutions were added to the reaction mixtures. The final reaction volumes were 2 mL. The reactions were carried out in closed glass vials on an orbital shaker at 50 °C with vigorous shaking. Water content of the reaction medium was measured by Karl-Fisher titration method. Corresponding water activity (a_w) was calculated using UNIFAC activity coefficient calculator v. 3.0 (*17*). The reverse micelles were created as follows: 2 mL of hexanol containing the activated substrate and different volumes of buffer (40–65 mL) with the dissolved enzyme were mixed with 1 mL of isooctane containing 0.3

M AOT. The degree of hydration of the system was quantified as a molar ratio of water and surfactant, *r*(water, surfactant). The mixed micelles were formed in similar way, only the isooctane contained not just AOT, but AOT/Tween or AOT/PEG combinations in different proportions, *i.e.* different mass ratios.

Analytical methods

Two methods for the analysis of substrate and product content in the reaction medium were applied, the HPLC and the spectrophotometric method. The HPLC system used was equipped with Knauer K-1001 pump (Berlin, Germany) and differential refractive index detector (RID) Shodex RI-71 (Munich, Germany). The column used was LiChrospher 100 RP-18 (4×250 mm, 5 μm) from Merck. The analyses proceeded under the following conditions: temperature at 25 °C, flow rate of 1 mL/min and the composition of the mobile phase acetonitrile/water ψ=25:75. A spectrophotometric method for measuring the total enzyme activity (hydrolytic+transglycosylation) developed by the research group of Hansson *et al.* (*15*) from Lund, Sweden, was slightly modified and adapted for the purpose of this research. The modifications were related to small differences in the reaction system and the type of spectrophotometer used. The quantity of depleted *p*-nitrophenyl-β-D-galactoside was calculated from the amount of liberated *p*-nitrophenol. The reaction was followed by measuring the increase of absorbance at 405 nm. The spectrophotometric system used was Varian Cary 50 Scan UV-Visible (Agilent Technologies, Palo Alto, CA, USA). The molar absorption coefficient for *p*-nitrophenol at 405 nm, pH=7.0 and 50 °C was 9.022 $mM^{-1} \cdot cm^{-1}$.

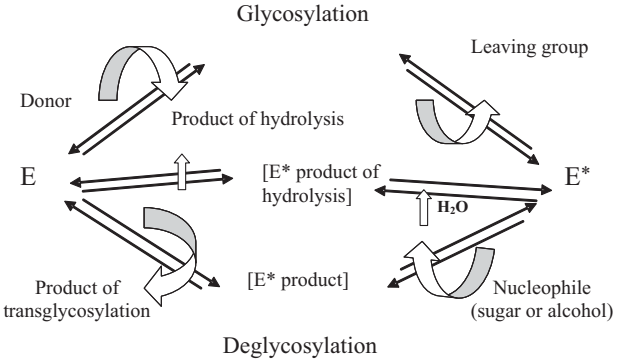
Protein concentration assay

The concentration of protein was measured by the method of Lowry using bovine serum albumin (BSA) as a standard (*23*).

Results and Discussion

Transglycosylation reaction was catalyzed by the *Aspergillus oryzae* β-galactosidase in hexanol as a medium. The *p*-nitrophenyl-β-galactoside was used as an activated donor. The reaction proceeded in two steps *via* a retention mechanism that is characteristic of the *Aspergillus oryzae* β-galactosidase, which belongs to the glycosidase family 1. In the first step, the glycosylation, the enzyme and the activated substrate formed the glycosyl-enzyme intermediate (Scheme 1).

In the second step, the deglycosylation, the glycosyl-enzyme complex reacts with the alcohol molecule and forms an alkyl-glycoside molecule, or with the water molecule (hydrolysis) and liberates a glycosyl group. The competition between these two reactions plays a crucial role in determining the final alkyl-β-glycoside yield and is quantified by the ratio of the two reactions, the r_S/r_H factor. The reaction system for hexyl-β-galactoside synthesis *via* a transglycosylation reaction catalyzed by *A. oryzae* β-galactosidase at the water activity level of 0.92 is presented in Fig. 1.



Scheme 1. The transglycosylation reaction of glycon donors catalyzed by the *Aspergillus oryzae* β-galactosidase *via* a two-step mechanism: (*i*) glycosylation, when the enzyme forms the glycosyl-enzyme complex and liberates the leaving group (*p*-nitrophenyl group), and (*ii*) deglycosylation, when the glycosyl-enzyme complex reacts either with the water molecule and liberates the sugar molecule (hydrolysis), or with another sugar molecule to form a disaccharide or with the alcohol molecule to form an alkyl glycoside

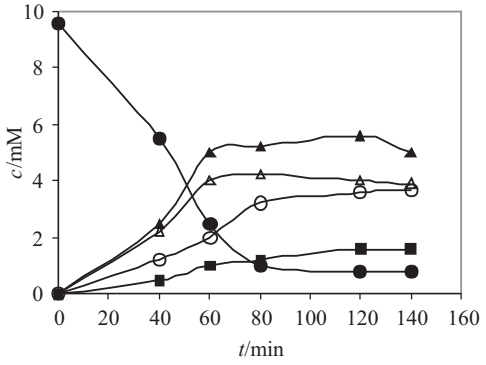


Fig. 1. Time course of the transglycosylation reaction in hexanol catalyzed by *A. oryzae* β-galactosidase at water activity a_w=0.92. The substrate *p*-nitrophenyl-β-galactoside (●) was converted to hexyl-β-galactoside (○) and galactose (▲), which was a sum of dissolved (■) and precipitated (△) galactose

Besides the fungal *A. oryzae* β-galactosidase, two other microbial β-galactosidases were investigated as catalysts for hexyl-β-galactoside synthesis, the bacterial *Escherichia coli* β-galactosidase and the yeast *Kluyveromyces marxianus* β-galactosidase. The r_S/r_H factors determined for the transglycosylation reactions catalyzed by the three mentioned glycosidases at the water activity level of 0.92 are presented in Table 1.

Table 1. The ratio of synthesis to hydrolysis (r_S/r_H) in transglycosylation reaction catalyzed by *A. oryzae* β-galactosidase, *K. marxianus* β-galactosidase and *E. coli* β-galactosidase by using *p*-nitrophenyl-β-galactoside as a substrate

a_w	*A. oryzae* β-galactosidase	*K. marxianus* β-galactosidase	*E. coli* β-galactosidase
0.60	**0.200**	0.090	0.045
0.92	**0.640**	0.150	0.267

As it can be seen, the *A. oryzae* β-galactosidase showed the highest value of r_S/r_H among the three examined galactosidases (r_S/r_H=0.64). This factor is practically a measure of the enzyme selectivity towards the aglycon donor and since its value is below 1, it means that hydrolysis and not synthesis is the dominating reaction. The selectivity of the enzyme is its intrinsic property, so the reaction conditions are to be optimized in order to achieve high yields in a reaction catalyzed by a certain enzyme.

In this work, besides the reaction system with free enzymes as catalysts, reverse micelles and mixed reverse micelles were examined as reaction systems for the transglycosylation reaction. Two activated substrates, *p*-nitrophenyl-β-glucoside and *p*-nitrophenyl-β-galactoside, were used as glycon donors. The reaction was carried out in hexanol/isooctane solvent mixture containing different volume fractions of water. The reverse micelles were formed when using AOT as a surfactant, while the mixed reverse micelles additionally contained cosurfactants such as Tween and PEG. The total enzyme activity as a function of the degree of hydration in the micelles was investigated. The achieved results are depicted in Fig. 2 and presented in Tables 2 and 3.

From the results presented in Fig. 2, it is obvious that the total enzyme activity per mg of protein of all three enzymes strongly depended on the degree of hydration in the reverse micelles. Thus, the *E. coli* β-galactosidase showed the highest value of the enzyme activity per mass of protein of 6.52 mmol/(min·mg), *A. oryzae* β--galactosidase of 1.80 mmol/(min·mg) and *K. marxianus* β-galactosidase of 1.46 mmol/(min·mg), all at the highest degree of hydration of *r*(water, surfactant)=12.04. The dependence of β-galactosidase/β-glucosidase ratio on the degree of hydration (Table 2) did not have the same pattern for all the enzymes examined, but varied a lot: from being slightly decreasing (for *K. marxianus* galactosidase) to being obviously increasing with the increase of the degree of hydration (for *E. coli* galactosidase and *A. oryzae* β-galactosidase). It is obvious that all tree enzymes have clearly expressed selectivity towards the glycon donor and that the β-galactosidase/β-glucosidase ratio is higher than 1 for all the examined cases, which means that galactose and not glucose is the preferring substrate for these enzymes (Tables 2 and 3). However, it can be suggested that generally both glucosidase and galactosidase activities are strongly dependent on the degree of hydration of the system and increase at its higher values.

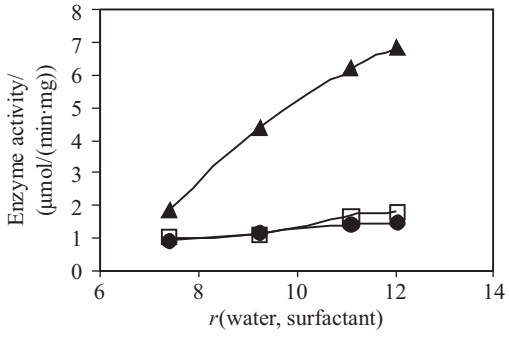


Fig. 2. Total activity as a function of the degree of hydration quantified as a molar ratio of water and surfactant (*r*) in simple reverse micelles created only by using AOT as a surfactant. The transglycosylation reaction was catalyzed by *E. coli* (▲), *A. oryzae* (□) and *K. marxianus* (●) β-galactosidases using *p*-nitrophenyl-β-galactoside as a substrate

Table 2. The ratio of β-galactosidase and β-glucosidase activities as a function of the degree of hydration in the simple micelles of *A. oryzae*, *K. marxianus* and *E. coli* β-galactosidase. Both *p*-nitrophenyl-β-galactoside and *p*-nitrophenyl-β-glucoside were used as activated substrates

r(water, surfactant)	*A. oryzae* β-galactosidase	*K. marxianus* β-galactosidase	*E. coli* β-galactosidase
	β-gal/β-glu		
7.40	1.61	1.91	1.22
9.26	1.23	1.71	1.18
11.11	1.62	1.62	1.52
12.04	**1.68**	1.63	1.58

Table 3. Characterization (specific β-galactosidase and β-glucosidase activities per mg of protein and the relationship between these activities) of different glycosidases

Enzyme	β-glucosidase	β-galactosidase	β-gal	Ref.
	U/mg		β-glu	
A. oryzae β-galactosidase*	1.07	1.80	**1.68**	this study
Sulfolobus solfataricus β-galactosidase	6.00	10.0	1.65	(*15*)
Caldocellum saccharolyticum β-glucosidase	6.00	4.00	0.64	(*15*)

*enzyme activity was determined as a total activity at *r*(water, surfactant)=12.04

These results correspond well with the data reported in the previously published papers that refer to both free and immobilized glycosidases (*16*,*24*). Thus, even Ljunger *et al.* (*25*) reported that the almond β-glucosidase is active only at water activity values higher than 0.6. Hansson *et al.* (*15*) went even further in the analysis of the effect of water activity on the total and synthetic activity of various free glycosidases from both wild types and genetically modified microorganisms. These researchers discovered that the water activity close to the level of saturation was the best for alkyl-β-glycoside synthesis in all the examined cases. They explained this phenomenon with the increased enzyme flexibility at higher water activity, flexibility that allowed not only a small water molecule, but also a bigger alcohol molecule to enter the glycosyl-enzyme complex.

As already mentioned, there is only little data reported on the effect of the degree of hydration on the activity of glycosidases entrapped in reverse micelles. Thus, Chen *et al.* (*26*) when synthesizing galactooligosaccharides in AOT/isooctane system found that the trans-

glycosylation reaction of the enzyme was strongly dependent on the molar ratio of water to surfactant.

However, there are many reports about the effect of the degree of hydration on the activity and the stability of lipases used for synthetic reactions in reverse micelles. Thus, Stamatis *et al.* (*20*) while investigating the effect of water content in reverse micellar bioreactor for esterification reactions catalyzed by *Pseudomonas cepacia* lipase discovered that in small micellar droplets, at very low *r*(water, surfactant) values, the enzyme is in its restricted configuration and is not capable of expressing its maximum activity. These researchers suggested that the lipase activity was optimum at medium degrees of hydration, when the size of micelles was small enough to permit the existence of only bound water molecules. Probably at higher *r*(water, surfactant) values, *i.e.* at a_w close to 1, the hydrolysis and not the synthesis might become the dominant reaction. Actually, lipases are generally known to be active at relatively low water activities since they act on hydrophobic substrates (*4*). Glycosidases, on the other hand, need more water for their activity (*16*).

From the results obtained in this work, it seems that glycosidases act similarly when used as free enzymes or when entrapped in reverse micelles, *i.e.* their activity increases with the increase of the degree of hydration in medium until the level of saturation is reached.

According to our observations the phase separation, *i.e.* the liberation of free water in the system occurs at *r*(water, surfactant) value of 13. From our previous work (*27*), it is known that when the phase separation occurs, the total reaction rate, *i.e.* the sum of hydrolysis and synthesis rates, suddenly increases to a great extent, but the rate of hydrolysis increases much more steeply than the rate of synthesis. It might be assumed that only when the reaction system persists in form of microemulsion, *i.e*. without separate water phase, the r_S/r_H factors for all three enzymes will have the highest values at the degrees of hydration close to the level of saturation of the solvent with water.

In order to allow the formation of larger sized reverse micelles, with more space for water entrapment, mixed reverse micelles, with different proportion of AOT/Tween and AOT/PEG were also created in the present work, but only with β-galactosidase from *Aspergillus oryzae*. The results achieved in these experiments are presented in Tables 4 and 5.

From the results presented in Table 4, it is noticeable that *A. oryzae* β-galactosidase entrapped in mixed reverse micelles created by a combination of the anionic surfactant AOT and the nonionic cosurfactant Tween, *i.e.* in mixed micelles AOT/Tween with a mass ratio ζ(AOT, Tween)=0.033 showed the highest total activity per mass of protein of 6.33 μmol/(min·mg). This activity was more than 3.5 times higher than the activity of the enzyme entrapped in the simple micelles. Similar results were obtained for the mixed micelles having lower mass ratios of AOT/Tween. It can be suggested that the mixed reverse micelles were larger in size than the simple ones (Scheme 2).

Therefore, when the enzyme was entrapped in mixed reverse micelles, it seemed that it had less chance to interfere with the AOT ionic head groups. It was assumed that these groups might have a negative effect on the enzyme activity and stability. In this way, in the mixed micelles, it seemed that an improvement of the interphase quality had been achieved, which resulted in an increase of the enzyme activity. It can also be suggested that in the larger size mixed micelles more water could be trapped, so the enzyme could be more active due to the higher conformational flexibility.

That the enzymes have higher activity and stability in mixed than in simple micellar systems is not a new statement. As already mentioned, there is not much data on glycosidases entrapped in mixed micelles, but there is little data about lipases in mixed microemulsion sys-

Table 4. Comparison of the efficiency of different types of reaction systems, the system with free enzyme, the simple micellar reactor and the mixed micellar AOT/Tween reactors, as potential bioreactors for transglycosylation reactions with *A. oryzae* β-galactosidase. The *p*-nitrophenyl-β-galactoside was used as a sugar substrate

Type of reverse micellar system	Total enzyme activity mmol/(min·mg)
free enzyme[1]	1.50
simple AOT micelles[2]	1.80
mixed AOT/Tween ζ(AOT, Tween)=0.033	**6.33**
mixed AOT/Tween ζ(AOT, Tween)=0.020	3.15
mixed AOT/Tween ζ(AOT, Tween)=0.014	2.79

[1]activity of free enzyme in the hexanol monophase system at water activity a_w=0.92

[2]activity of enzyme entrapped in the simple micelles at a degree of hydration of *r*(water, surfactant)=12.04

degree of hydration was calculated on the basis of AOT

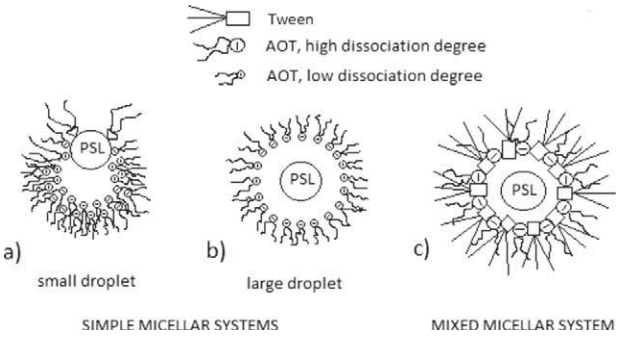
Table 5. The transglycosylation activity of *A. oryzae* β-galactosidase in mixed reverse micelles of the type AOT/PEG. The *p*-nitrophenyl-β-galactoside was used as a sugar substrate

Type of reaction system	Total enzyme activity mmol/(min·mg)
free enzyme[1]	1.50
simple AOT micelles[2]	1.80
mixed micelles AOT/PEG ζ(AOT, PEG)=10	1.90
mixed micelles AOT/PEG ζ(AOT, PEG)=1	**2.70**
mixed micelles AOT/PEG ζ(AOT, PEG)=0.5	2.63

[1]activity of free enzyme in the hexanol monophase system at water activity a_w=0.92

[2]activity of enzyme entrapped in the simple micelles at hydration degree of *r*(water, surfactant)=12.04

degree of hydration was calculated on the basis of AOT



Scheme 2. The model of simple AOT micelles and mixed reverse micelles that besides the anionic AOT surfactant contained Tween as a nonionic cosurfactant: a) in very small sized simple micellar droplets with low volumes of water the *Aspergillus oryzae* β-galactosidase (AOG) was probably present in the vicinity of the interphase and was affected by the polar AOT head groups; b) the enzyme was probably localized in the centre of the large droplets of simple micelles with high amounts of water trapped (higher degree of hydration of the system); c) the proposed localization of AOG in the mixed micelles of the type AOT/Tween (it was suspected that the Tween molecules had neutralizing effect on the AOT polar head groups)

tems (*28*,*29*). In both publications it was reported that the addition of nonionic cosurfactants increased the enzyme activity and stability compared to the activity and stability measured in simple reverse micelles that were formed using only the anionic AOT surfactant. Arcos *et al*. (*28*) investigated the stability of *Pseudomonas* sp. lipase in simple AOT and in mixed (AOT/Tween) reverse micelles and found that the latter system was much more convenient for lipase utilization in organic media. The lipase stability in mixed micelles was higher than the stability of lipase entrapped in the simple micelles, in all examined cases. These researchers explained this phenomenon with the fact that the Tween molecules increased the droplet size of the micelles and reduced the lipase--interfacial interaction. It seemed that the lipase-interface interaction was stronger in smaller droplets and was detrimental for the enzyme stability and activity. Stamatis *et al.* (*20*) reported that the partitioning of the substrates among the micellar water pool, the micellar interface and the organic phase of the microemulsion system influenced the enzyme activity to a great extent and that the addition of nonionic cosurfactants increased the enzyme activity and changed the partitioning coefficients of the compounds present in the different phases. Yamada *et al.* (*30*) also reported increased activity of *Chromobacterium viscosum* lipase in AOT/Tween and AOT/Triton mixed micelles compared to its activity in simple reverse micelles. These researchers suggested that the mentioned nonionic cosurfactants suppressed the electrostatic and hydrophobic interactions between the surfactant AOT and the lipase, and in that way affected the enzyme activity. Chiang (*29*) examined the activity of *Candida rugosa* lipase in AOT reverse micellar system and found that the addition of nonionic cosurfactant, tetraethylene glycol dodecyl ether ($C_{12}E_4$) had a very positive effect on the enzyme activity.

In the present work other types of mixed micelles were also prepared, which contained PEG as a cosurfactant. The results are presented in Table 5.

It can be seen that the *A. oryzae* β-galactosidase in all the prepared mixed micelles containing PEG in different proportions to AOT showed higher activity compared to the activity in simple micelles. In all the three cases, the enzyme activity was higher than the activity of the enzyme entrapped in simple micelles. Thus, the enzyme entrapped in the mixed micelles AOT/PEG with a mass ratio ζ(AOT, PEG)=1 had 1.50 times higher activity than the enzyme in simple micelles.

That the PEG molecules have a positive effect on the enzyme activity and stability has already been reported. Thus, Talukder *et al.* (*31*) while analyzing the activity and stability of *Chromobacterium viscosum* lipase in AOT/PEG reverse micelles discovered that the addition of low molecular mass PEG (M_r=400) diminished the negative effect of the AOT-lipase interaction and preserved the enzyme activity and stability even at very high concentrations of AOT in the system. The enzyme was two times more stable in AOT/PEG microemulsion systems than in the simple micellar ones. Hayashi *et al*. (*32*), while examining the behaviour of lipases in the reaction system for olive oil hydrolysis, noticed the same positive effect of the addition of PEG on the lipase activity in AOT/isooctane reverse micelles. Arcos *et al*. (*28*) suggested that the nonionic alcohols increased the fraction of neutralized AOT head groups and in this way decreased their deleterious effect on the enzyme activity and stability.

Conclusions

Both simple and mixed micellar reaction systems could be effective as bioreactors for the transglycosylation reaction catalyzed by the examined microbial galactosidases. As suspected, the glycosidases acted in the same way in all so far investigated reaction systems: their activity increased with the increase of the degree of hydration in the micellar systems. All three enzymes had strongly expressed β-galactosidase activity in simple micellar reactors, similarly to previous statements that referred to the enzymes used as freely dispersed preparations in unconventional organic media. The mixed reverse micelles, which besides the main anionic AOT surfactant additionally contained nonionic cosurfactants such as Tween and PEG, showed excellent performances and affected the enzyme activity in the most positive way.

This work is another contribution that confirms the glycosidase behaviour in unconventional reaction media. What is even more important, it proved that the very popular microemulsion reaction systems, simple and mixed, until today almost exclusively employed for the reactions catalyzed by lipases, could be very successfully used for application of glycosidases in unconventional reaction media.

Acknowledgements

The author would like to thank professor Slobodanka Kuzmanova from the Faculty of Technology and Metallurgy in Skopje, Republic of Macedonia, for all the help and support given during the research presented in this paper, and also professor Patrick Adlercreutz for all the given advice and opinions during the study at the Department of Biotechnology, at the Center for Chemistry and Chemical Engineering in Lund, Sweden. The finan-

cial support from the Ministry for Education and Science of the Republic of Macedonia is also gratefully acknowledged.

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