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Lactose hydrolysis in an aqueous two-phase system by whole-cell β-galactosidase of *Kluyveromyces marxianus*: partition and separation characteristics

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Abstract

The selection of suitable aqueous two-phase system for lactose hydrolysis by whole-cell β-galactosidase is described. Partitioning of substrate, products, biocatalyst and viable, permeabilized and glutaraldehyde-treated cells of *Kluyveromyces marxianus* in various systems containing polyethylene glycol and dextran was studied. Some characteristics of the selected systems were also established.

1. Introduction

The utilization of sweet whey is an important economical and ecological problem in cheese manufacture [1]. This drawback can be solved by hydrolysis of lactose, which is the major component of whey. Various methods of lactose hydrolysis are used [1,2], and hydrolysis of lactose in an aqueous two-phase system (ATPS) has shown promising results [3,4].

Bionconversion in an ATPS is an interesting alternative to the use of immobilized biocatalysts or membrane bioreactors. This method offers several advantages, *e.g.*, efficient mass transfer, biocompatibility, stability of biocatalyst and simultaneous separation of product [5,6]. Biocatalysis in an ATPS can be easily realized in a semi-continuous or continuous mode and integrated with other purification techniques [7,8]. However, it is necessary to find a system that has favourable partitioning (biocatalyst, substrate, product) and separation characteristics for each individual bioconversion process.

In this paper, the partitioning behaviour of viable and treated cells of *Kluyveromyces marxianus*, β-galactosidase, lactose, glucose and galactose in various polyethylene glycol (PEG)–dextran (Dx) ATPSs and the separation characteristics of selected systems are described.

2. Experimental

2.1. Organisms

The yeasts used in this investigation were *Kluyveromyces marxianus* CCY eSY2 and *K. marxianus* CCY 51-1-1 obtained from the Czecho-Slovak Collection of Yeasts, Bratislava, Slovak Republic. Stock cultures were maintained

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on lactose-based agar at 5°C and subcultured monthly.

2.2. Materials

PEG 6000 was obtained from NCHZ (Nováky, Slovak Republic) and PEG 20 000 from LOBA Feinchemie (Austria).

Dextrans D 40, D 70, D 250, D 500 were a kind gift from Biotika (Slovenská Ľupča, Slovak Republic).

Boehringer Mannheim Biochemica tests for the determination of lactose, glucose, galactose were used.

o-Nitrophenyl-β-D-galactopyranoside (ONP-G), *o*-nitrophenol (ONP) and other chemicals of analytical-reagent grade were purchased from Lachema (Brno, Czech Republic).

2.3. Whole-cell yeast β-galactosidase preparation

The yeast biomass was prepared by aerobic cultivation in lactose-based medium [60 g l^{-1} lactose, 5 g l^{-1} yeast extract, 5 g l^{-1} $(NH_4)_2SO_4$ and 2 g l^{-1} $(NH_4)_2HPO_4$]. After the cultivation the cells were centrifuged (3000 *g*, 10 min), washed three times with cold tap water and suspended in 0.05 *M* potassium phosphate buffer (pH 6.5) to a concentration of 100 g l^{-1} (dry mass). The cells were permeabilized by the addition of 15% (v/v) of chloroform–ethanol (1:9) at 25°C for 15 min [9]. The solvents were removed by centrifugation and the cell ghosts were washed with buffer. Finally, this suspension was treated with 0.4% (w/v) glutaraldehyde at 5°C for 10 min and washed with cold buffer [10].

2.4. Partitioning experiments

The cells (viable, permeabilized and glutaraldehyde treated) were added to the tubes with various ATPS at a concentration of 10 g l^{-1}. The tubes were vigorously shaken for 2 min to allow the phases to homogenize. After sufficient separation (2 h), the numbers of cells in both phases were determined microscopically.

The partitioning of free β-galactosidase, lactose, glucose and galactose was measured after centrifugal separation of the phases (1500 *g*, 4 min). The partition coefficient was defined as the ratio of the concentrations (cells, saccharides) or activity (β-galactosidase) in the top and bottom phases.

All partitioning experiments were carried out at pH 6.5 and 25°C. The samples were measured in triplicate.

2.5. Characteristics of phase systems

The phase systems were prepared in 0.05 *M* phosphate buffer (pH 6.5) from a stock solution of the phase polymers. The systems were mixed intensively with a magnetic stirrer and allowed to settle in a test-tube at 25°C. The percentage of dextran-rich bottom phase dispersed in the PEG-rich top phase was determined turbidimetrically and by centrifugation of the top phase [6,8]. After phase separation, top-to-bottom volume ratio was also measured. The effect of the yeast cells on the phase separation rate and the volume ratio was investigated in the concentration range 1–40 g l^{-1}.

2.6. Analytical methods

β-Galactosidase activity was measured with the chromogenic substrate ONPG [9] at 25°C. A 0.05-ml volume of the sample was added to 0.45 ml of 4 m*M* ONPG solution in 0.05 *M* potassium phosphate buffer (pH 6.5) with 0.1 m*M* $MnCl_2$. The reaction was stopped by adding 1 ml of 0.2 *M* Na_2CO_3 after 4 min and the absorbance at 416 nm was measured. The molar absorbtivity for ONP of 4600 l mol^{-1} cm^{-1} was determined under the same conditions. One unit of β-galactosidase activity was defined as the amount of enzyme required to release 1 μmol of ONP per minute.

Glucose, galactose and lactose concentrations were determined by using kits prepared by Boehringer Mannheim Biochemicals.

3. Results and discussion

3.1. Partition of viable and treated cells

For semi-continuous or continuous extractive bioconversion, minimizing the removal of biocatalyst with the product-containing phase is crucial. Hence extremely one-sided partitioning of the biocatalyst is required. This is easy to accomplish for cells, but much more difficult for soluble enzymes [5,6]. Some workers [9,10] have described the use of whole-cell β-galactosidase preparations, which could be more advantageous than using free enzymes for lactose hydrolysis in ATPSs. Moreover, the whole-cell biocatalysts often exhibit higher operational stability and their preparation is easier and cheaper.

First the partitioning behaviour of intact cells of two strains of lactose utilizing the yeast *K. marxianus* in various PEG–dextran systems was investigated. Some partition coefficients are given in Table 1. As expected, the cells preferred the dextran-rich bottom phase. The cell partitioning was more extreme with increasing polymer molecular mass and concentration. Only the partition coefficients of *K. marxianus* CCY 51-1-1 increased with increase in the dextran molecular mass. Such behaviour of the yeast cells in an ATPS can be explained by the strong affinity of cell walls containing polysaccharides (glucans, mannans) to the more hydrophilic dextran phase. Although there are not many data about partitioning of yeast cells or cell walls, similar results have been reported for baker's yeasts, *Saccharomyces cerevisiae* and *Candida boidinii* [6,11].

Table 2
Partitioning of the permeabilized cells of *K. marxianus* CCY eSY2 in PEG 20 000–dextran (6:4, w/w) systems

Dextran	Partition coefficient
D 40	$<10^{-6}$
D 70	$<10^{-6}$
D 250	$<10^{-6}$
D 500	$<10^{-6}$

As the best results were obtained in ATPSs containing PEG 20 000, these systems were used in the following experiments. In addition, separation of products from high-molecular-mass PEG with ultrafiltration, which is a potential method for product separation from the polymer, is easier.

Permeabilization of the cells with solvents (described under Experimental) did not affect the distribution (Table 2). Probably the surface properties of the cells, which determine their partitioning, were not changed by this operation. Under the experimental conditions the solvent

Table 1
Partitioning of the viable cells of *K. marxianus* CCY eSY2 and CCY 51-1-1 in various PEG–dextran systems

PEG (%, w/w)	Dextran (4%, w/w)	Partition coefficient	
		K. marxianus CCY eSY2	*K. marxianus* CCY 51-1-1
6% PEG 20 000	D 40	$<10^{-6}$	$<10^{-6}$
	D 70	$<10^{-6}$	$<10^{-6}$
	D 250	$<10^{-6}$	$<10^{-6}$
	D 500	$<10^{-6}$	$<10^{-6}$
6% PEG 6000	D 70	$1.5 \cdot 10^{-2}$	$2.0 \cdot 10^{-4}$
	D 250	$5.4 \cdot 10^{-4}$	$5.0 \cdot 10^{-3}$
	D 500	$8.9 \cdot 10^{-4}$	$6.1 \cdot 10^{-3}$
4% PEG 20 000	D 250	$7.9 \cdot 10^{-4}$	$3.1 \cdot 10^{-4}$
	D 500	$4.9 \cdot 10^{-4}$	$7.6 \cdot 10^{-4}$

only modified the membrane composition, which resulted in the removal of diffusion barriers for small molecules, such as lactose, glucose and galactose [9,12]. Stabilization of β-galactosidase inside the cells with glutaraldehyde cross-linking also did not have any effect on the partitioning behaviour of the cells (Table 3).

3.2. Partitioning of free enzyme and saccharides

As partial release of β-galactosidase from treated cells during the long-term hydrolysis process could occur [9,10], it is important to establish the partitioning behaviour of the free enzyme. The partitioning of soluble β-galactosidase from *K. marxianus* CCY eSY2 was interesting (Table 4). This enzyme preferred the dextran-rich phase directly with increase in PEG concentration. A similar behaviour of yeast β-galactosidase in the PEG–pullulan system and mould β-galactosidase in PEG–salt systems has been described [3,4]. In contrast, β-galactosidase from *Escherichia coli* exhibited affinity to the PEG phase in PEG–salt systems [4,13]. The structure and properties of β-galactosidases differ according to their producing microorganisms and therefore it is difficult to predict the partitioning behaviour of the enzyme in ATPSs [4].

The partitioning of the saccharides (lactose, glucose and galactose) is shown in Table 5. As expected [7], partition coefficients of about 1 were found. Hence a shift of the reaction equilibrium in these systems is improbable.

3.3. Phase separation characteristics

In addition to partitioning of the biocatalyst, substrate, product, some properties of the ATPS used, such as the volume ratio of the phases and the separation time of the system, play important roles in extractive bioconversion.

The volume ratios of the phases of the systems considered are given in Table 3. Generally, a high volume ratio of the top to the bottom phase is suitable when the biocatalyst prefers the bottom phase, because a higher yield of product is obtained in the opposite phase.

The separation characteristics of the selected systems are shown in Fig. 1. The system PEG 20 000–dextran 40 (8:4, w/w), which exhibits a high volume ratio and slow phase separation, was selected for semi-continuous lactose hydrolysis because a high product yield is the most important factor in this instance. In contrast, rapid phase separation is needed for the continu-

Table 4
Partitioning of β-galactosidase from *K. marxianus* CCY eSY2 in PEG 20 000–dextran D 40 systems

PEG 20 000–dextran D 40 (w/w)	Partition coefficient
4:8	$5.9 \cdot 10^{-3}$
6:6	$1.7 \cdot 10^{-3}$
8:4	$1.2 \cdot 10^{-4}$

Table 5
Partitioning of saccharides in PEG 20 000–dextran D 40 two-phase systems

PEG 20 000–dextran (w/w)	Partition coefficient		
	Glucose	Galactose	Lactose
4:8	0.99	0.88	0.91
6:6	0.94	0.91	0.91
8:4	1.06	0.97	0.88

Table 3
Partitioning of the stabilized cells of *K. marxianus* CCY eSY2 in PEG 20 000 systems

PEG 20 000–dextran D 40 (w/w)	Partition coefficient	Volume ratio (top/bottom phase, ml/ml)
4:8	$<10^{-6}$	10.1/9.9
6:6	$<10^{-6}$	13.0/7.0
8:4	$<10^{-6}$	16.1/3.9

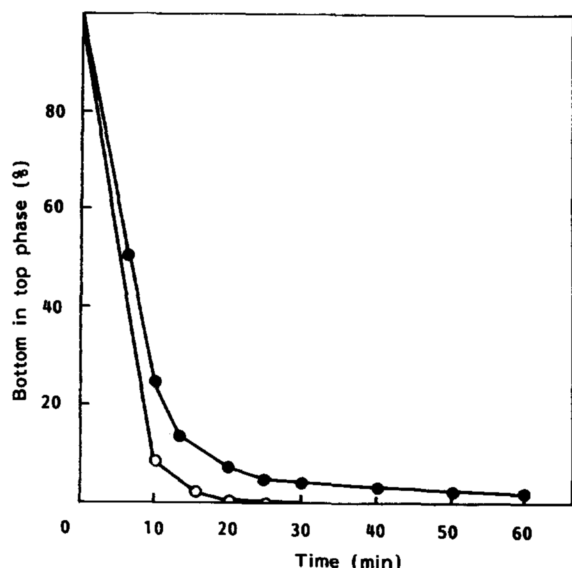

Fig. 1. Phase separation characteristics at 25°C and pH 6.5 of PEG 20 000–dextran D 40 (●) 8:4 (w/w); (○) 4:8 (w/w).

ous process. Slow phase separation lowers the volume productivity and requires a large volume of a settler. Therefore, the system PEG 20 000–dextran D 40 (4:8, w/w) is more suitable for the continuous process.

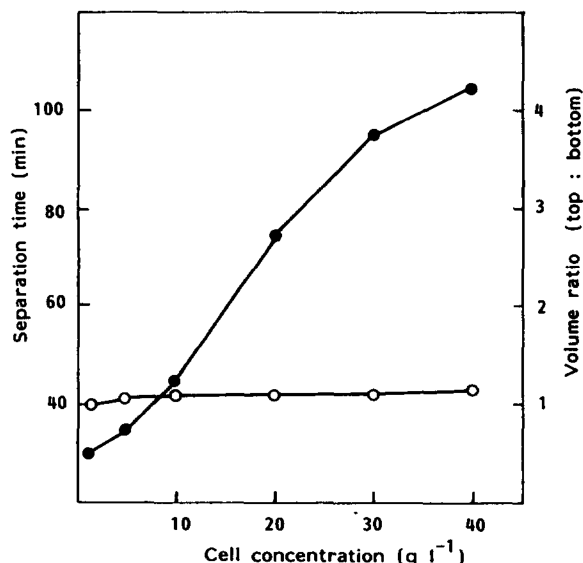

Fig. 2. Effect of treated cells of *K. marxianus* on (●) the separation time and (○) the phase volume ratio of the system PEG 20 000–dextran D 40 (4:8, w/w) at 25°C and pH 6.5.

The presence of cells is known to influence both the volume ratio of the phases and the separation time [6]. The effect of the whole cell concentration is illustrated in Fig. 2. The volume ratio was affected negligibly in the concentration range considered, but the separation time was affected significantly. Hence some limitation of lactose hydrolysis in the ATPS occurs at high biocatalyst concentrations.

In conclusion, the selection of suitable ATPSs for lactose hydrolysis with whole-cell yeast β-galactosidase and some its characteristics have been reported We are now investigating the semi-continuous and continuous processes of lactose hydrolysis in the selected ATPSs.

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