Biotechnology and Bioprocess Engineering **2004**, 9: 147-150

Permeabilization of *Ochrobactrum anthropi* SY509 Cells with Organic Solvents for Whole Cell Biocatalyst

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Abstract Permeabilization is known to overcome cell membrane barriers of whole cell biocatalysts. The use of organic solvents is advantageous in terms of cost, simplicity, and efficiency. In this study, *Ochrobactrum anthropi* SY509 was permeabilized with various organic solvents. Treatment with organic solvents resulted in lower permeability barriers due to falling out lipids of the cell membrane. Therefore, permeabilized cells showed higher enzyme activity with no cell viability. Among various organic solvents, 0.5% (v/v) chloroform was selected as the most efficient permeabilizing reagent. Changes in the cell membrane structure were observed and the residual amounts of phospholipids of the cell membrane were measured to investigate the mechanism of the improved permeability.

Keywords: permeabilization, permeability barrier, organic solvent, *Ochrobactrum anthropi*, phospholipids

INTRODUCTION

The use of whole cell biocatalysts over purified enzymes is advantageous in terms of cost, isolation, and stability. When substrates cannot permeate sufficiently enough through the cell membrane of the cell biocatalysts, permeabilization treatment is required. Permeabilized cells are able to provide a source of biocatalysts similar to that of immobilized enzymes [1].

There are various methods of permeabilization, such as use of surfactants, sonication, freezing and thawing [1-3]. Among them, the use of organic solvents is known to be most effective because of the low cost and simple procedure. When organic solvents come in contact with the cell membrane, the structure of the cell membrane is altered [4] such that the substrate can freely enter and reach the desired enzymes in the periplasmic space. The representative organic solvents that enhance cell membrane permeability are toluene, chloroform, ethanol, diethyl ether, and dimethyl sulfoxide [5-7]. Moreover, permeabilization with the organic solvents causes the microorganisms to become unviable. Thus, permeabilization can additionally reduce unwanted side reactions and minimize energy loss for cell growth.

In this work, *Ochrobactrum anthropi* SY509 was permeabilized with organic solvents to obtain dead cell biocatalysts for denitrification. Denitrification is the process of forming nitrogen (N_2), the intermediate nitric oxide (NO), and nitrous oxide (N_2O) from nitrate or nitrite [8]. Each step of this process is catalyzed by an enzyme system. This enzyme system is composed of nitrate reductase and nitrite reductase located in the periplasmic and/or inner membrane [9-10]. In this study, the most efficient organic solvent for the permeabilization was selected to prepare whole cell biocatalyst showing increased activities of denitrifying enzyme system. In addition, we examined the permeabilized cell membrane structure and determined the residual amount of phospholipids contained in the cell membranes to elucidate the mechanism of the permeabilization. The results presented here could be used to understand cause and effect of the permeabilization.

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MATERIALS AND METHODS

Materials

The organic solvents used for permeabilization were toluene, chloroform, isopropyl alcohol, acetone, ethanol, and dimethyl sulfoxide obtained from Sigma (St. Louis, MO, USA). And other reagents were of analytical grade.

The microscopic analyses were carried out using a FE-SEM (JSM-6700F model) and a TEM (JEM1010 model).

Permeabilization Procedure

Ochrobactrum anthropi SY509 was used as a model microorganism. The cells were grown and harvested us-

ing the same method as described previously [11]. The washed cell pellet was resuspended in a potassium phosphate buffer (80 mM, pH 7.0) and the organic solvent was added to the cell suspension (100 g DCW/L) to permeabilize the cells. Various solvents were used for permeabilization: toluene, chloroform, isopropyl alcohol, acetone, ethanol, and dimethyl sulfoxide. After the suspended cells were incubated at 4°C for 15 min [5], the permeabilized cells were recovered by centrifugation at 12,000 rpm for 20 min and were washed twice with a potassium phosphate buffer before used for denitrification reaction.

Enzyme Activity and Cell Viability Assay

The nitrate and nitrite reductase activity was measured following published methods [12,13]. The assay mixture contained cells (optical density at 660 nm: 1.0), a potassium phosphate buffer (80 mM, pH 7.0), 1 mM of benzyl viologen as an electron donor, and 10 mM of sodium dithionite. Benzyl viologen is reduced by sodium dithionite, subsequently giving electrons to the denitrifying enzymes. The mixture was stirred, and the reaction was initiated by adding 10 mM potassium nitrate. After 10 min, nitrate reduction was stopped by vortexing. Sodium dithionite and benzyl viologen were oxidized, so that they were unable to give out more electrons. The cells were removed by centrifugation, and the concentrations of nitrate were measured using ion-chromatography.

To measure cell viability [14], the permeabilized and control cells (optical density at 660 nm: 5.0) were suspended in mixture containing a potassium phosphate buffer (80 mM, pH 7.0) and 10 mM of glucose as an electron donor. The reaction was started with the addition of 10 mM of potassium nitrate. After 1 h of the reaction, the cells were removed and the concentrations of nitrate were measured in the same manner as the enzyme activity assay.

Lipid Extraction and Direct Estimation of Phospholipids

Due to is suitability for cell suspensions, the method used by Bligh and Dyer [15] was employed to extract lipids from the permeabilized and control cells. To a 1 mL sample cell suspension, 3.75 mL of a mixture containing chloroform and methanol (1:2) was added and vortexed for 15 min. Then, 1.25 mL of chloroform and 1.25 mL of water were added in that order. After centrifugation of the sample mixture, two liquid phases were separated. The upper phase was discarded and the lower phase was collected. The lipid extract was obtained after the lower phase was evaporated.

The amount of phospholipids in the lipid extract was estimated using the simple colorimetric method. This method is based on the complex formation between phospholipids and ammonium ferrothiocyanate [16]. First, the lipid extract obtained was dissolved in 1 mL chloroform. Then 0.5 mL of ammonium ferrothiocyanate reagent (27 g of ferric chloride and 30 g of ammonium thiocyanate in 1 L water) was added. After vortexing for 1 min, the mixture was centrifuged. A red lower layer formed after centrifugation was collected. The absorbance of this solution at 488 nm represents the relative amount of phospholipids in the mixture.

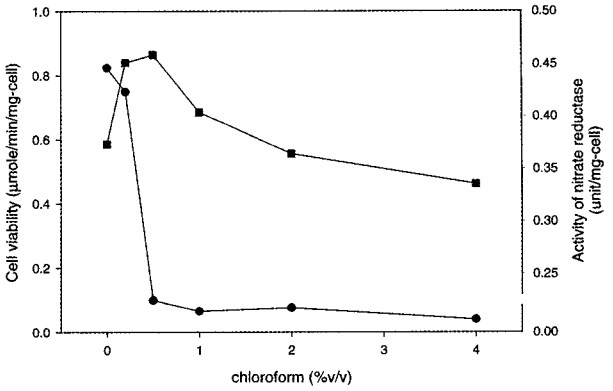

Fig. 1. Effect of chloroform concentration on viability of *Ochrobactrum anthropi* SY509 and nitrate reductase activity.

RESULTS AND DISCUSSION

Permeabilization with Organic Solvents

After the cells were permeabilized with various organic solvents, enzyme activity and cell viability were examined to select the most efficient organic solvent for the biocatalyst. Several organic solvents were selected as permeabilizing reagents for *Ochrobactrum anthropi* SY509 cells from previous reports [1,5-6,17-19].

The effect of chloroform concentration on permeabilization is shown in Fig. 1. The range of chloroform concentration was determined from previous studies [5,17]. When the cells were treated with more than 0.5% (v/v) chloroform, cell viability was almost zero as shown in Fig. 1. Moreover, the nitrate reductase activity of the permeabilized cells increased with increasing chloroform concentration of up to 0.5% (v/v). Therefore, permeabilization with 0.5% (v/v) chloroform was the most efficient for higher enzyme activity.

Besides chloroform, other organic solvents, such as toluene, isopropyl alcohol, acetone, ethanol, and dimethyl sulfoxide, were tested. The optimal concentrations of each solvent were also determined using the above-mentioned method (data not shown). According to the results, 2% (v/v) toluene, 20% (v/v) isopropyl alcohol, 40% (v/v) acetone, 40% (v/v) ethanol and 40% (v/v) dimethyl sulfoxide were determined to be the most suitable concentrations, respectively.

When the nitrate reductase activity of the cells was compared with different solvents, the cells permeabilized with chloroform showed the highest activity (Table 1). These results showed that 0.5% (v/v) chloroform, in the case of *Ochrobactrum anthropi* SY509 cells, was most efficient among the tested solvents.

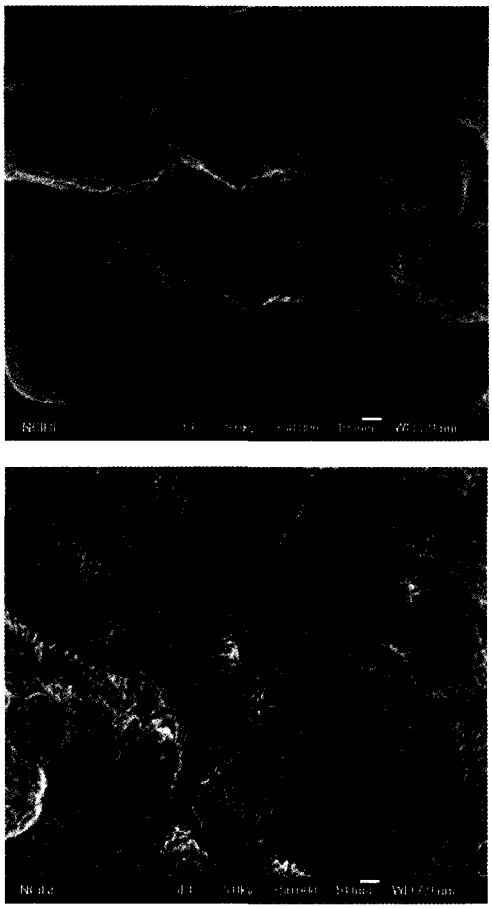

Fig. 2. Scanning electron micrographs of *Ochrobactrum anthropi* SY509 (a) control cells, (b) permeabilized cells with 0.5% (v/v) chloroform.

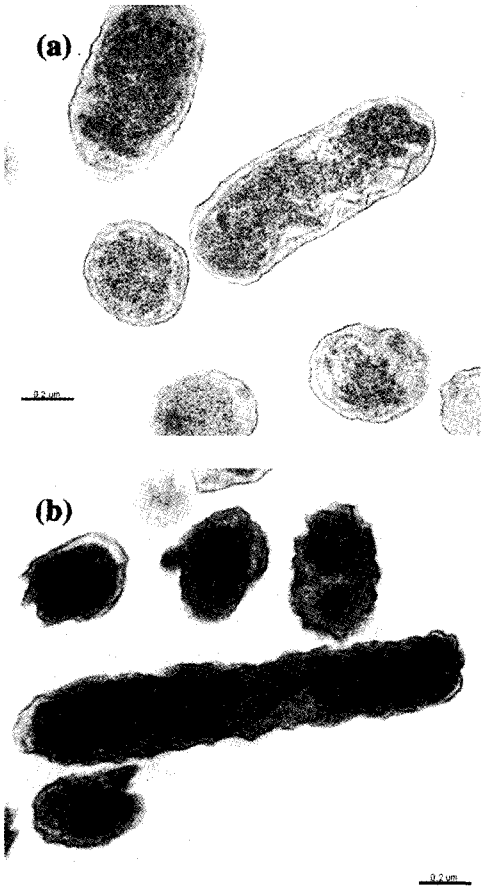

Fig. 3. Transmission electron micrographs of *Ochrobactrum anthropi* SY509 (a) control cells, (b) permeabilized cells with 0.5% (v/v) chloroform.

Effect of Permeabilization on the Structure of Cellular Outer Membrane

Ochrobactrum anthropi SY509 is a gram-negative bacterium that has a membrane structure composed of an outer membrane, periplasmic space, and inner membrane. Enzymes related to denitrification are known to exist mostly in periplasmic space [9,10]. When the cells are treated with organic solvents, the outer membrane becomes disorganized. To confirm this effect, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used after the cells were treated with 0.5% (v/v) chloroform. Fig. 2(a) shows that the control cells had smooth intact surfaces. On the other hand, the cells treated with 0.5% (v/v) chloroform for 15 min showed rough altered surface, as shown in Fig. 2(b). As shown in TEM (Fig. 3), the variations in the membrane structure were detected in comparison to the control cells. These observations revealed that the outer membrane was changed in such a way that the permeability barrier was decreased, yet the overall morphology of the cells was maintained. Therefore, the substrate could more easily access the enzyme contained in periplasmic space without the release of intracellular enzymes [18].

Effect of Permeabilization on the Composition of the Cell Membrane Lipids

When the cells were treated with organic solvents, the outer cell membrane changed to have lower permeability. It has been suggested that this was due to falling out lipids of the cell membrane [20]. The fundamental structure and function of cell membranes depend on the type and content of lipids. And a lipid bilayer of the outer membrane is mainly made up of phospholipids [21]. Thus, the residual amount of phospholipids was determined and compared between the control and the permeabilized cells. Table 1 shows that the permeabilized cells had relatively less phospholipids than the untreated cells. Although there was no linear correlation between enzyme activity and the amount of residual phospholipids, it could be supposed that the high enzyme activity was somewhat attributable to the loss of cell membrane lipids. Organic solvents dissolved off cell membrane lipids that the permeability barrier was decreased resulting in the

Table 1. Effect of different organic solvents on nitrate reductase activity and comparison of residual phospholipids after permeabilization

Organic solvents	Optimal concentration (%, v/v)	Nitrate reductase activity (unit/mg-cell)	Residual phospholipids (% of control)
Control	-	0.34	100
Toluene	2	0.42	66.10
Chloroform	0.5	0.43	63.29
Isopropyl alcohol	20	0.33	77.15
Acetone	40	0.40	75.36
Ethanol	40	0.39	73.74
Dimethyl sulfoxide	40	0.33	78.44

※ The 1 unit of nitrate reductase will produce 1 μmole of nitrate-N per min.

higher enzyme activity.

In this report, we showed that permeabilization to cause higher enzyme activity and a more nonviable state - minimizing energy loss for cell growth - in *Ochrobactrum anthropi* SY509. 0.5% (v/v) chloroform was determined to be the most effective permeabilizing solvent. Furthermore, the change in cell membrane structure and the residual amount of phospholipids were also examined to elucidate the mechanism of permeabilization.

This whole cell biocatalyst with a less permeable barrier can be used for more efficient denitrification reactions. Further, if electron donors are supplied, electrons can reach the enzymes directly without undergoing complicated electron transfer pathways [22]. Therefore, denitrification can be achieved without carbon sources such as glucose. Because of this advantage, permeabilized cells can be applied to electrochemical denitrification systems.

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[Received November 7, 2003; accepted March 27, 2004]