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Optimization of Catechol Production Using Immobilized Resting Cells of *Pseudomonas putida* in Aqueous/organic Two-phase System

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An aqueous/organic two-phase reaction system was applied to the production of catechol using immobilized resting cells of *Pseudomonas putida* CY400. Water/ethyl ether system was used because of high partition coefficient of catechol and thus to reduce the product inhibition and degradation. Among the tested immobilization carriers, polyacrylamide gel gave the highest catechol productivity. The immobilization seemed to protect the cells against solvent toxicity. From the simulation of reaction conditions based on two-phase models, it was found that there was an optimum acetate concentration at fixed benzoate and cell concentrations for the catechol productivity. A lower phase volume ratio (lower fraction of organic phase) gave a higher productivity. However, the substrate conversion was low at low phase volume ratio.

Catechol and its derivatives are useful in the production of polymerization inhibitor, agricultural chemicals and synthetic flavors (19). Catechol has been implicated as an intermediate in the microbial degradation of aromatic compounds in several genera of soil bacteria, particularly *Pseudomonas* (9). The microbial or enzymatic routes for the production of catechol from aromatic compounds such as phenol, toluene, benzene, and benzoate have already been reported (10, 14, 18). In the previous study (7), a catechol-accumulating mutant of *Pseudomonas putida* was developed and used for the production of catechol in a medium containing benzoic acid. It was also reported that a biotransformation using resting cells was efficient for the catechol production. Use of resting cells was desirable because it provides higher concentration of catechol and requires less amount of energy source than use of growing cells. Additionally it obviates the danger of contamination.

In many biotransformations, the accumulation of product in reaction media adversely affects the bioconversion through product inhibition. The environments of biocatalysis can give the possibility of the product degradation. Attempts avoiding these problems have been tried: vaccum fermentation, dialysis fermentation, and use of adsorbents (1, 17). Robinson *et al.* reported the productivity of 3-methylcatechol from toluene using *P. putida* was improved by 3~4 folds by using activated carbon (16). Extraction with nonaqueous solvents provides a method for the recovery of products from bioconversion reactions. Several studies have been investigated on the extractive bioconversion using organic solvents (4, 21).

In the present study, an aqueous/organic two-phase system was used in the catechol production to reduce dioxygenase inhibition by catechol and the product degradation. Two-liquid phase biocatalysis is particularly advantageous when the organic solvent is inhibitory and denaturing on biocatalysts such as enzymes and microbial cells. The use of immobilized cells in solvent system is a method of reducing molecular toxicity of organic solvents (11).

In the present study, mathematical models, previously developed for the aqueous/organic two-phase reaction system (6), were applied to the resting-cell biotransformation system (7). The effects of various operation parameters and design factors on the reaction performance in the two-phase system were evaluated using the kinetic models.

MATERIALS AND METHODS

Microorganism and Media

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A catechol accumulating mutant strain, *P. putida* CY 400 (7) was used for the production of catechol from sodium benzoate. The strain was maintained on nutrient agar plates and incubated in LB media (5 g/l yeast extract, 5 g/l tryptone and 10 g/l NaCl in distilled water). The production medium specially designed for catechol production under resting cell conditions (7) contained 4.25 g KH_2PO_4, 3.15 g $Na_2HPO_4 \cdot 12H_2O$, 0.1 g $MgSO_4$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.024 g $FeSO_4 \cdot 7H_2O$, 0.009 g $MnSO_4 \cdot H_2O$, 0.006 g $ZnSO_4 \cdot 7H_2O$, 0.004 g $CoSO_4 \cdot 7H_2O$ and distilled water to make 1 liter. The production media were formulated without nitrogen source to induce resting cell conditions. The initial pH was adjusted to 7.5 by 1 N NaOH.

Bacterial Cultivation

Seed cultures were performed in 500 ml Erlenmeyer flasks, filled with 100 ml of LB media and shaken at a speed of 300 rpm. The cultivation temperature was 30°C. Fermentor cultures were performed in a 2 liter-jar fermentor with a working volume of 1 liter. The cells grown in the seed culture for 18 h were used for inoculation to the jar fermentor. Aeration and agitation rates were maintained at 2 vvm (l/l·min) and 300 rpm, respectively.

Immobilization Methods

Cells that were grown up in the fermentor using LB media, were harvested by centrifugation (8,000 g, 10 min, 4°C). After washing with phosphate buffer (50 mM, pH 7.0), followed by re-centrifugation, the cells were suspended in the production media containing sodium benzoate (3 g/l) to induce benzoate 1,2-dioxygenase activity. Various carriers (5 g dry weight) were contacted with the cells suspended in distilled water in a shaking incubator for 12 h at 30°C. The tested adsorption carriers included activated carbon, celite and glass bead. Ion exchange and adsorption resins such as Diaion® UBK530, HPK25, FRK101, SP206 and Amberlite® XAD-7 were also tested as immobilization carriers. The cells were also entrapped in calcium alginate (15), κ-carrageenan (15), polyvinyl alcohol (20) and polyacrylamide gels (15). The immobilization procedure using polyacrylamide gel is shown as follows: The solution of acrylamide (30%), N,N'-methylene-bisacrylamide (2%) and N,N,N',N'-tetramethylethylene-diamimle (TEMED, 1%) were mixed with cell broth suspended in the production media to a volume fraction of 1 : 1. Potassium persulfate (0.25%) was added to the above mixture placed on ice bath. The pH of acrylamide solution was adjusted to 7.5 before use. The plate-type gel formed was placed on a standard mesh (No.14), and pressed by a plastic plate to be cut into beads of uniform size (15).

Reaction System

A Lewis cell-type reactor (6) was used for the two-phase system, as illustrated in Fig. 1. The reactor (12 cm in diameter, 25 cm in height) was immersed in a temperature-controlled water bath. Reaction media were mixtures of ethyl ether and the aqueous production media containing sodium acetate and sodium benzoate. Immobilized cells of *P. putida* were suspended in the aqueous media to start the production of catechol. The total working volume of the reaction was 1 liter and the reaction temperature was 30°C. Both phases were stirred by two six-bladed impellers (5 cm diameter, 1 cm height) to maintain a quiescent interface. Air was supplied into the aqueous phase at a rate of 10 cm^3/sec.

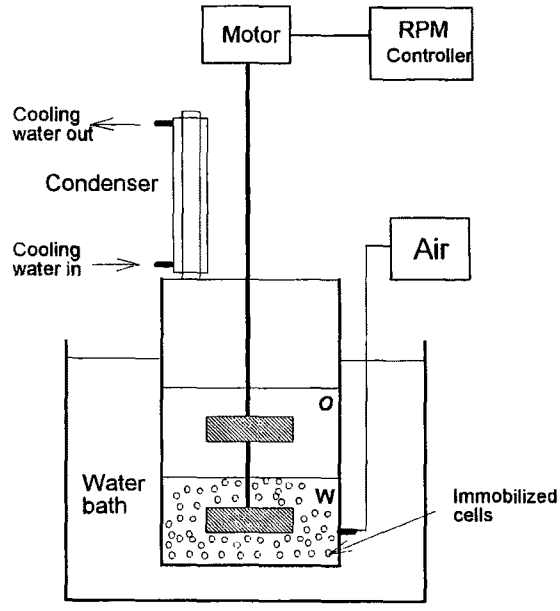

Fig. 1. Schematic diagram of the two-phase reactor for the catechol production using immobilized resting cells of *P. putida*. W, aqueous phase; O, organic phase.

Analytical Methods

The cell concentration was measured by determining the absorbance of the cultures at 660 nm using a spectrophotometer (Uvikon 930, Kontron Instrument). The concentrations of acetate and benzoate were measured after esterification (5) by Gas Chromatography (HP5890, Hewlett Packard Co.) using FID detector with HP-1 capillary column. Catechol was determined by the method of Arnow (2). Spectrophotometric assays were carried out with the sonicated cell-free extracts to measure *in vitro* activities of enzymes involving the bioconversion: catechol 1,2-dioxygenase (EC 1.13.11.1, C12O) and benzoate 1,2-dioxygenase (EC 1.13.99.2, B12O) according to the methods as previously described (7).

Chemicals

Sodium acetate and sodium benzoate were purchased from Junsei Chemical (Tokyo, Japan). Acrylamide and N,N'-methylene-bisacrylamide were obtained from Sigma Chemical (St. Louis, U.S.A.) and other reagents were of analytical grade.

Table 1. Effects of carrier type and immobilization method on catechol production.

Immobilization method	Carrier	Immobilized cell loading per carrier (g/g)	Catechol productivity (g/g·h)
Adsorption	activated carbon	0.026	ND[a]
	celite	0.022	6.21e-5
	porous glass bead	0.031	7.48e-5
	Diaion SP206	0.028	1.32e-5
	Amberlite XAD-7	0.035	2.34e-5
Ionic binding	Diaion UBK530[b]	0.024	9.54e-5
	Diaion HPK25[b]	0.017	1.92e-5
	Diaion FRK101[b]	0.024	ND
Entrapment	calcium alginate gel		7.00e-5
	κ-carrageenan gel		8.00e-5
	polyvinylalcohol		3.33e-5
	polyacrylamide gel		8.33e-4

[a]not detected. [b]treated with polyethyleneimine.

RESULTS AND DISCUSSION

Immobilization

First various adsorption carriers were tested (Table 1). Glass bead showed a relatively high catechol productivity per unit weight of carrier. However, the adsorption method possesses considerable drawbacks: inadequate strength of cell retention and limited quantity of adsorption. Ion exchange resins treated with polyethyleneimine (3) were used as cell carriers because they generally have higher porosity. However, the adsorbed cells by ionic binding were desorbed by the competition with ionic components of reaction media such as phosphate and sodium salt. Among the tested entrapment methods, the polyacrylamide entrapment method showed the highest catechol productivity. This method was very simple, and various shapes of catalyst could be molded. Moreover, the cell loading and bead size were easy to control. Polyacrylamide was reported to have non-ionic character and little effect on pH dependence of the biocatalysts (12). Unfortunately the catechol production was delayed 6~7 h compared with free cells after immobilization by polyacrylamide. This is probably due to the fact that *P. putida* is one of gram-negative bacteria and subjected to the toxicity of the monomer during immobilization. The immobilization can give a protecting environment to the cells against the other toxic compounds as organic solvent used in the two-phase system (11). In addition, the entrapment of cells by polyacrylamide gel was advantageous because it avoided direct contact of the cells with organic solvent through the two-liquid interface.

Model Approaches

Mathematical models for the production of catechol from benzoate using *P. putida* CY400 in the aqueous phase have already been proposed (7). A set of two-phase reaction models has also been developed, based on partition and mass transfer phenomena (6). Both modeling concepts were combined to formulate mathematical models for the catechol production in two-phase system, as summarized in Table 2.

It was assumed that the cell concentration is approximately constant under resting cell conditions (dX/dt =0). The acetate and benzoate uptake rates and catechol degradation rate were formulated based on Michaelis-Menten type equations. The biosynthesis rate of benzoate 1,2-dioxygenase (B12O) was induced by benzoate (8), and repressed by acetate, that was considered in E-

Table 2. Model equations for the catechol production in two-phase reaction media.

Compound/ Enzyme	Equations in the aqueous phase		Equations in the organic phase	
Acetate	$\frac{dA_W}{dt}=\frac{k_A A}{V_W}(A_O-m_A A_W)-q_A X$, where $q_A=\frac{q_{max}A_W}{K_{EA}+A_W}$	(1)	$\frac{dA_O}{dt}=-\frac{k_A A}{V_O}(A_O-m_A A_W)$	(2)
Benzoate	$\frac{dB_W}{dt}=\frac{k_B A}{V_W}(B_O-m_B B_O)-\nu_1 E_1$, where $\nu_1=\left(\frac{k_1 B_W}{K_{M1}+B_W+B_W^2/K_{BI}}\right)\left(\frac{1}{1+C_W/K_{CI}}\right)$	(3)	$\frac{dB_O}{dt}=-\frac{k_B A}{V_W}(B_O-m_B B_W)$	(4)
Catechol	$\frac{dC_W}{dt}=\frac{k_C A}{V_W}(C_O-m_C C_W)+\left(\frac{M_C}{M_B}\right)\nu_1 E_1-\nu_2 E_2$, where $\nu_2=\frac{k_2 C_W}{K_{M2}+C_W}$	(5)	$\frac{dC_O}{dt}=-\frac{k_C A}{V_O}(C_O-m_C C_W)$	(6)
B12O	$\frac{dE_1}{dt}=\alpha X-k_{D1}E_1$, where $\alpha=\alpha_{max}\left(\frac{B_W}{K_{E1}+B_W}\right)\left(\frac{1+aA_W^n}{1+aA_W^n(1+b)}\right)$	(7)		
C12O	$\frac{dE_2}{dt}=\beta X-k_{D2}E_2$, where $\beta=\beta_{max}\frac{C_W}{K_{E2}+C_W}$	(8)		

Initial conditions: $A_W=A_{Wi}$, $B_W=B_{Wi}$, $A_O=B_O=C_W=C_O=E_1=E_2=0$ at t=0.

quation 7 using a three-parameter model (7). The formation of catechol-degrading enzyme (C12O) was induced by catechol (Equation 8). Since the immobilized cells were present in the aqueous phase only, biosynthesis models of the enzymes were defined only in the aqueous phase (Equation 7 and 8).

Determination of the Kinetic Parameters

The partition coefficients of acetate, benzoate and catechol between the aqueous and organic phases were obtained from separate experiments at 30°C without the cells, and the values are shown in Table 3. Also the mass transfer coefficients of the compounds were estimated from the experimental data obtained in the two-phase reactor according to the method in the previous study (6).

The parameters (q_{max}, K_{EA}, k_1, K_{M1}, k_2 and K_{M2}) of Michaelis-Menten type equations were estimated using reciprocal plots such as q_A vs. 1/A (data not shown). The parameters (a, b and n) of catabolite repression in Equation 7 and 8 were estimated by nonlinear regression using experimental data. The Marquardt-Levenberg algorithm (13) was used to estimate the parameter values. This algorithm seeks the values of the parameters that minimize the sum of the squared differences between the observed values and the predicted values of the dependent variable (catechol productivity). Some parameters such as α_{max}, β_{max}, K_{E1}, k_{D1}, K_{E2}, k_{D2} were obtained by curve-fitting. Some parameters of the two-phase system, for example parameters involved in acetate consumption (q_{max} and K_{EA}), substrate inhibition (K_{BI} and K_{CI}) and enzyme synthesis (α_{max} and β_{max}) were quite different from those of the aqueous phase reaction, as shown in Table 4. The other parameters in the aqueous phase reaction (7) were used for the two-phase system with the same value.

Because of solvent toxicity, the catechol production was highly inhibited. Maximum specific acetate consumption rate (q_{max}) and maximum specific enzyme synthesis rates (α_{max} in B12O and β_{max} in C12O) were estimated as greatly reduced values in the two-phase models. Kinetic constants such as K_{EA}, K_{CI} and K_{BI} were slightly increased by the immobilization, probably due to the diffusion limitation and protecting effect induced by the immobilization. The model equations were numerically integrated by Runge-Kutta method using Matlab® with Simulink subroutine (The Mathworks, Inc., U.S.). The model profiles were in a measure of agreement with experimental data as shown in Fig. 2.

Table 3. Partition coefficients and mass transfer coefficients of substrates and product for water-ethyl ether system.

Parameter	Parameter value[a]
m_A (-)	0.25
m_B (-)	0.48
m_C (-)	6.40
k_A (cm/h)	11.4
k_B (cm/h)	3.18
k_C (cm/h)	5.34

[a]at 150 rpm.

Table 4. Comparison of kinetic parameters between the aqueous phase and two-phase reactions.

Parameter[a]	Type of reaction media and cells	
	Aqueous phase, free cells	Aqueous/organic two-phase, immobilized cells
q_{max} (h^{-1})	0.065	0.0048
K_{EA} (g/l)	0.5	0.7
K_{CI} (g/l)	1.2	1.96
K_{BI} (g/l)	7.5	9.75
α_{max} (U/g·h)	25.4	4.57
β_{max} (U/g·h)	0.6	0.11

[a]parameters whose values were changed according to the introduction of organic solvent.

Reaction Kinetics in Two-phase System

Acetate and benzoate dissolved in the reaction media hardly diffused into the organic phase because the partition coefficients of the two substrates were very low. Acetate and benzoate are poorly soluble in the organic solvent, which was rather advantageous because they should be utilized by the cells present in the aqueous phase only. Produced catechol was partitioned to the organic solvent phase, which minimized the induction of the catechol-degrading enzyme (C12O) and inhibited the catechol-producing enzyme (B12O). These are potential

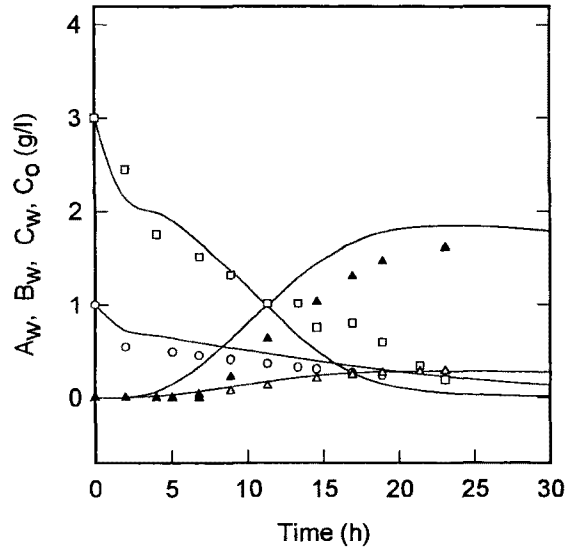

Fig. 2. Comparison of model profiles and experimental data for the two-phase system using immobilized cells (A_{wi}=1, B_{wi}=3, X_{wi}=10 g/l, V_W=V_O=0.5 l and A=113 cm^2).
Symbols and lines represent experimental and simulation data, respectively. ○, A_W; □, B_W; △, C_W; ▲, C_O.

advantages of introduction of a two-phase system to the catechol production. However, the catechol producing activity was lower than that in the aqueous phase reaction (7) because of the solvent toxicity.

The kinetic behaviors of the single (aqueous) phase system and two-phase system were compared in Fig. 3. The time courses of acetate and benzoate concentrations showed quite different profiles in both systems. In the single phase system, the catechol production as well as the disappearance of substrates occurred rapidly (Fig. 3, a-c). However, the produced catechol was decayed to ring-fission products by C12O on successive incubation in the aqueous phase. In the two-phase system, the produced catechol was transferred into the organic phase, and it lowered the product degradation. The partition of the product into the organic phase and the reduced induction of the catechol-degrading activity (C12O) resulted in higher product stability. The C12O activity was remained at low levels since the concentration of the inductive product (catechol) was low in the aqueous phase.

Simulation Study

Simulation study on batch-type operation was performed using various reaction conditions such as initial acetate (A_{wi}) and benzoate (B_{wi}) concentrations and phase volume ratio ($\varphi=V_o/V_w$) (Fig. 4~6). The substrate conversion and productivity were expressed in terms of total mass in both phases as follows:

$$x = \frac{V_O B_{Oi} - (V_O B_O + V_W B_W)}{V_O B_{Oi}} \times 100(\%)$$

and

$$P = \left[\frac{V_O C_O + V_W C_W}{V_t \cdot t} \right] \quad (g/l \cdot h)$$

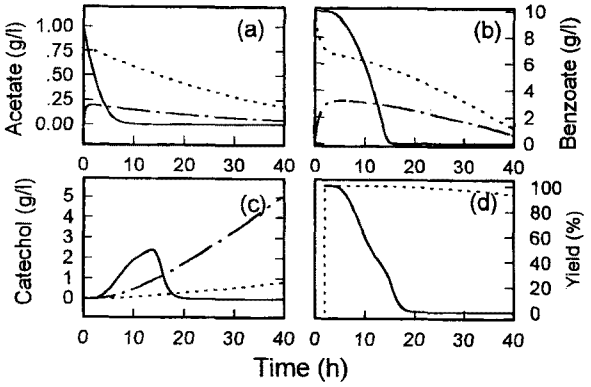

Fig. 3. Comparison of model profiles for the single phase and two-phase system using immobilized cells (A_{wi}=1, B_{wi}=3, $X_{imm}=X_{free}$=10 g/l, $V_w=V_O$=0.5 liter, A=113 cm^2).
Lines represent simulation data: (——) aqueous phase in single phase system, (·····) aqueous phase in two-phase system, (– – –) organic phase in two-phase system.

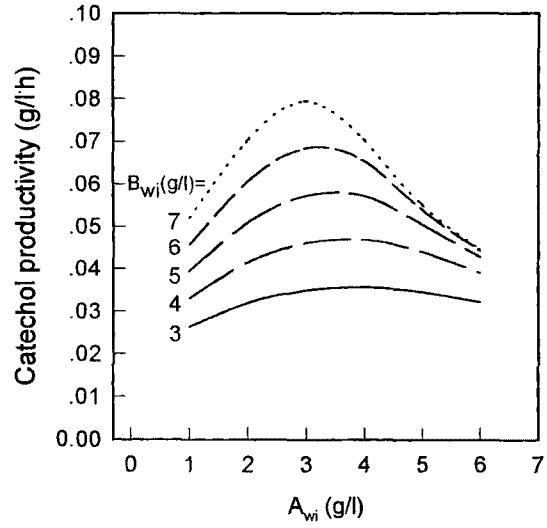

Fig. 4. Effects of the initial acetate concentration on the catechol productivity and conversion at various initial benzoate concentrations (X_{imm}=30 g/l, $V_w=V_O$=0.5 liter and A=113 cm^2).
Lines represent simulation data.

In the aqueous phase reaction (7), the lower the acetate concentration, the higher the catechol productivity was. However, there was an optimum value (approximately 3~3.5 g/l) of acetate concentration for the catechol production in the two-phase reaction (Fig. 4). When the cells were cultivated on high concentration of acetate (over the optimum value), the catechol-producing activity was repressed. This might be as a result of catabolite repression when using easily metabolized carbon source such as acetate (7). At low levels of acetate, the catechol productivity increased with increasing acetate concentration. This range does not seem under the control of catabolite repression, but the control of mass (acetate) transfer limitation, due to low concentration of acetate.

As shown in Fig. 5, the catechol productivity increased with increasing benzoate concentration, which is similar to the case of aqueous phase reaction catalyzed by free cells (7). It appeared that there was a broad optimum value in the benzoate concentration for the catechol production.

In this system, the mass transfer of catechol, which is inhibitory to B12O activity and subject to the degradation by C12O, was very important. A phase volume ratio giving a higher product transfer rate was advantageous to this system. As previously discussed (6), a lower phase volume ratio can lead a higher product transfer rate. The toxicity of organic solvent on the cells depends on the phase volume ratio. However, the solvent toxicity was not reflected in the proposed models in the present study. In Fig. 6, the effect of phase volume ra-

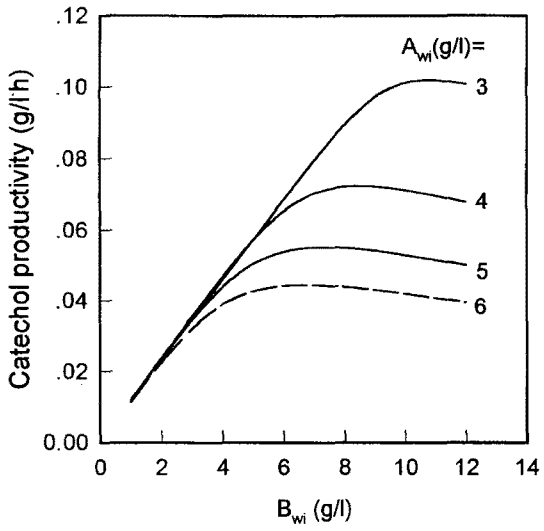

Fig. 5. Effects of the initial benzoate concentration on the catechol productivity and conversion at various initial acetate concentrations (X_{imm}=30 g/l, V_W=V_O=0.5 liter and A=113 cm^2). Lines represent simulation data.

tio on the catechol productivity was presented without toxicity consideration. It showed that lower phase volume ratio (lower fraction of organic phase) gave higher productivity. However, the substrate conversion was low at low phase volume ratio. It was probably due to the reduced biocatalytic reactivity with diluted substrate concentration in the aqueous phase, as previously discussed (6).

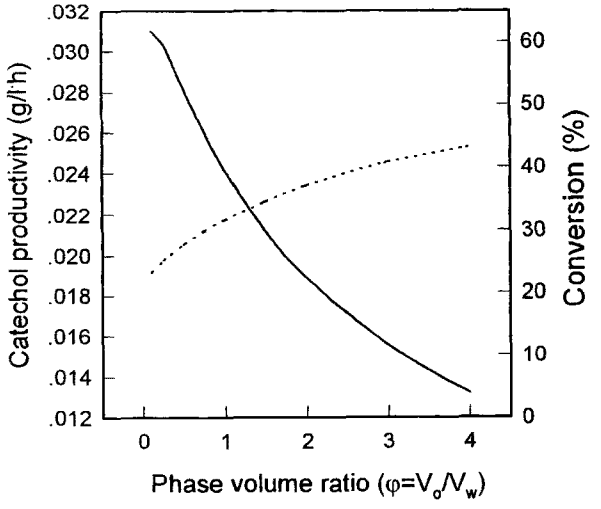

Fig. 6. Effects of phase volume ratio on the reaction performance of two-phase system (X_{imm}=30, A_{wi}=3, B_{wi}=6 g/l, V_T= 1 liter and A=113 cm^2).
Lines represent simulation data: (——) catechol productivity, (·····) conversion.

NOTATION

A : Acetate concentration in aqueous (or organic) phase (g/l)
B : Benzoate concentration in aqueous (or organic) phase (g/l)
C : Catechol concentration in aqueous (or organic) phase (g/l)
E_1 : Benzoate 1,2-dioxygenase (B12O) activity (U/l)
E_2 : Catechol 1,2-dioxygenase (C12O) activity (U/l)
K_{E1} : Constant of E1 (B12O) synthesis rate equation (g/l)
K_{E2} : Constant of E2 (C12O) synthesis rate equation (g/l)
K_{EA} : Constant of acetate consumption rate equation (g/l)
K_{M1} : Michaelis-Menten constant of B12O activity (g/l)
K_{M2} : Michaelis-Menten constant of C12O activity (g/l)
K_{BI} : Inhibition constant of benzoate consumption rate equation (g/l)
K_{CI} : Inhibition constant of catechol consumption rate equation (g/l)
k_1 : Reaction rate constant of B12O activity (g/U·h)
k_2 : Reaction rate constant of C12O activity (g/U·h)
k_B : Mass transfer coefficient of benzoate (cm/h)
k_C : Mass transfer coefficient of catechol (cm/h)
k_{D1} : Specific B12O degradation rate constant (h^{-1})
k_{D2} : Specific C12O degradation rate constant (h^{-1})
M_B : Molecular weight of benzoate (g/mol)
M_C : Molecular weight of catechol (g/mol)
m_A : Equilibrium partition coefficient of acetate (dimensionless)
m_B : Equilibrium partition coefficient of benzoate (dimensionless)
m_C : Equilibrium partition coefficient of catechol (dimensionless)
P : Productivity (g/l·h)
q_A : Specific acetate consumption rate (h^{-1})
q_{max} : Maximum specific acetate consumption rate (h^{-1})
S : Substrate (benzoate)
t : Reaction time (h)
V : Volume of aqueous (or organic) phase (l)
V_T : Total volume of reaction (l)
X : Cell concentration (g/l)
x : Substrate conversion (%)
α : Specific B12O formation rate (U/g·h)
α_{max} : Maximum specific B12O formation rate (U/g·h)
β : Specific C12O formation rate (U/g·h)
β_{max} : Maximum specific C12O formation rate (U/g·h)
ν_1 : Specific reaction rate of B12O activity (g/U·h)
ν_2 : Specific reaction rate of C12O activity (g/U·h)
φ : Phase volume ratio, φ=V_o/V_w (dimensionless)

Subscript

free : Free cells
i : Initial state
imm : Immobilized cells

o : Organic phase
w : Aqueous phase

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