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Flow injection analysis system for monitoring of succinic acid in biotechnological processes

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Abstract

In this study, a flow injection analysis (FIA) system using a cartridge of immobilized isocitrate lyase (ICL) and isocitrate dehydrogenase (ICDH) was developed to monitor the concentrations of succinic acid in biotechnological processes. The ICL and ICDH immobilized on VA-Epoxy Biosynth E3-carrier had a good operational lifetime (up to 24 h) and storage stability (up to 30 days). The FIA system with the immobilized ICL/ICDH cartridge was characterized with respect to the factors affecting the activity of the immobilized enzymes, such as pH of carrier solution, temperature, sample matrix, etc. Optimal pH value of the immobilized enzymes was slightly shifted in the alkaline range, i.e. 9.0. Some components such as 10 g l^{-1} lactose, 3 g l^{-1} malate and 3 g l^{-1} oxaloacetate in sample solution had significant activating effects (more than 10%) on the response of the FIA system. But the activity of the immobilized ICL and ICDH was not largely influenced by some components like imidazole (1 mM), sodium azide (10 mM) and semicarbazide (2 g l^{-1}) added to carrier buffer solution. The FIA system with an enzyme cartridge was applied to on-line monitor the concentrations of succinic acid in a continuously stirred reactor and a fermentation process of immobilized *Escherichia coli*, and showed good sensitivity and reliability of the FIA system developed in this work.

Keywords: Flow injection analysis; Succinic acid; Isocitrate lyase; Isocitrate dehydrogenase; Biotechnological processes

1. Introduction

Succinic acid is produced in plants, animals and microorganisms and has extensive application in industry making foods, pharmaceuticals and cosmetics [1]. A few studies have recently focused on the production of succinic acid by fermentation of microorganisms [2,3]. A few strains like *Escherichia coli*, *Anaerobiospirullum succinicproducens* have been studied and developed for the mass production of succinic acid [4]. Succinic acid has been also used for the production of enzymes by a microorganism like *Pseudomonas fluorescens* as substrate [5]. For the development of biotechnological processes with high product yield, it is necessary to monitor and control the concentrations of succinic acid.

Concentration of succinic acid in biological samples has been usually determined by a few methods such as gas chromatography [6], high performance liquid chromatography (HPLC) [7] and capillary electrophoresis [8]. However, these methods are expensive and time-consuming, so that a few enzymatic methods have been suggested and used for the analysis of succinic acid in foodstuffs.

Succinic dehydrogenase (SDH) converts succinic acid to fumaric acid in the presence of NADH, but due to its unstability it is difficult to purify the enzyme and to use it for the determination of succinic acid [9]. Succinyl-CoA synthetase (SCS) is commercially available and has been used to determine the concentrations of succinic acid in biological samples. SCS is combined with pyruvate kinase (PK) and lactate dehydrogenase (LDH) in the presence of coenzyme A (CoA) and inosine 5′-triphosphate (ITP), phosphoenolpyruvate (PEP) and NADH. The consumption of NADH, measured by the change in fluorescence intensity at 340 nm (excitation)/440 nm (emission), has been correlated to the concentration of succinic acid [10].

In a biotechnological process, the concentration of succinic acid changes with time, that an analysis technique with high specificity and rapidity is required to monitor the concentration of succinic acid on-line. The flow injection

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analysis (FIA) using an enzymatic method can meet this demand well [11]. However, it is not economical to apply the enzymatic method including SCH or SCS to a FIA system, because some chemicals like CoA, ITP and NADH are very expensive.

Succinic acid reacts with glyoxylate to form isocitrate in the presence of the isocitrate lyase (ICL) in glyoxylate cycle [12]. The isocitrate formed is then oxidized to 2-oxoglutarate by $NADP^+$ in the presence of isocitrate dehydrogenase (ICDH) in the following reactions path:

$$\text{succinic acid} + \text{glyoxylate} \xrightarrow{\text{ICL}} \text{isocitrate}$$

$$\text{isocitrate} + NADP^+ \xrightarrow{\text{ICDH}} \text{2-oxoglutarate} + CO_2 + NADPH + H^+$$

From the above reactions, the concentration of succinic acid is correlated to the amounts of NADPH produced, which is measured by a fluorometer at an excitation wavelength of 340 nm and an emission wavelength of 440 nm. This enzymatic reaction can be applied to a FIA technique, because enzymes used in this reaction can be easily obtained and only $NADP^+$ is necessary as cofactor of the reaction.

Based on this reaction, a FIA system has been developed by immobilizing the enzymes, i.e. ICL and ICDH on controlled pore glass (CPG) and determined the concentration of succinic acid in foodstuffs [13]. However, the system was not used for a biotechnological process, where the concentrations of succinic acid vary with time and have to be monitored continuously. To apply a FIA system to a biotechnological process, it should have high stability and good adequacy on a process. Due to easy immobilization technique and increased stability, a few enzymes have been immobilized on epoxy polymeric supports [14,15]. In this work, the ICL and ICDH were immobilized on epoxy-polymer supports for the on-line monitoring of succinic acid in biotechnological processes. Therefore, the objective of this work is to investigate the characteristics of the ICL and ICDH immobilized on epoxy-polymer supports systematically and to apply the FIA system to on-line monitor the concentration of succinic acid in biotechnological processes.

2. Experimental

2.1. Chemicals and reagents

Isocitrate lyase (EC.4.1.3.1, from *Bacillus stearothermophilus*) and isocitric dehydrogenase (EC.1.1.1.42, from Porcine Heart) were purchased from Sigma Co. (Seoul, Korea). Na-NADP was obtained from Hoffman LaRoche Co. (Basel, Switzerland). Polymer carrier support (VA-Epoxy-Biosynth E3) was purchased from Fluka Co. (Buchs, Switzerland) and small cartridges (1 ml) for packing of carrier support were obtained from Mobitec Co. (GÖttingen, Germany). All other chemicals were of analytical reagent grade and purchased from Sigma Co. and Fluka Co.

2.2. Immobilization of enzymes

ICL (5 units) and ICDH (100 units) were immobilized separately on epoxy carrier support as reported previously [15]. The immobilized ICL was first packed into a cartridge (1 ml) and then the immobilized ICDH. A separation filter (Mobitec Co.) was inserted between the immobilized ICL and ICDH. The cartridge with two immobilized enzymes was rinsed with 0.1 M phosphate buffer (pH 7.0) and equilibrated for the analysis. The enzyme cartridge was then stored at 4 °C until use.

2.3. Flow injection system

The immobilized enzyme reactor was integrated into the FIA system as shown in Fig. 1. After injecting samples into the carrier stream the $NADP^+$ solution was introduced for 50 s into the carrier solution. The $NADP^+$ solution dissolved in distilled water was mixed with samples before the enzyme cartridge and then reacted in the immobilized enzyme cartridge. The difference between the baseline of a peak and its maximum height was defined as the flu-

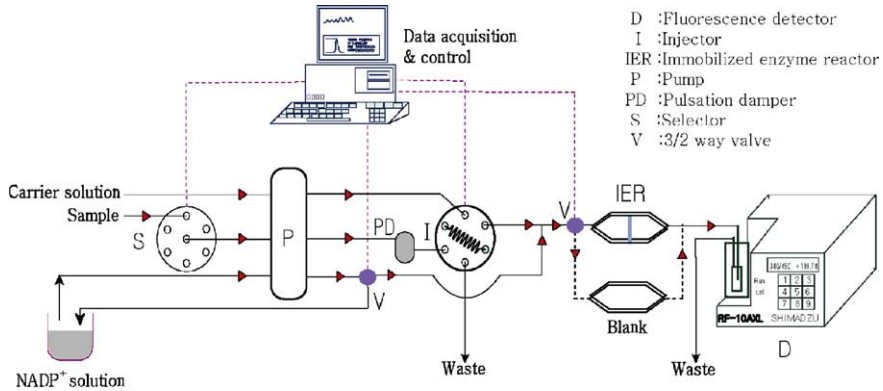

Fig. 1. Schematic diagram of the FIA system for the monitoring of succinic acid with an immobilized ICL and ICDH cartridge.

Table 1
Reference operating conditions of the FIA system

Parameters	Values
Carrier flow rate (ml min^{-1})	1.5
Sample	
Flow rate (ml min^{-1})	0.3
Injection volume (μl)	125
Temperature (°C)	Room temperature
Activity of enzymes immobilized (U)	5 (ICL) 100 (ICDH)
$NADP^+$ solution	
Concentration (mM)	1.0
Flow rate (ml min^{-1})	1.0
Addition time (s)	50
Cycle time (s)	360

Standard carrier buffer solution consisted of 4.35 g l^{-1} K_2HPO_4, 1.8 g l^{-1} NaH_2PO_4, 3.0 g l^{-1} NaCl, 5 mM glyoxylate, 0.1 mM cysteine and 1.32 mg l^{-1} hydrazine at pH 9.0. Enzyme unit reprsesents the amount of enzymes added to initially to epoxy carrier support.

orescence intensity measured under some operating conditions of the FIA system. The intensity of fluorescence emitted by NADPH was measured at an excitation wavelength of 340 nm and an emission wavelength of 440 nm by a spectrofluorometer (Shimadzu Co., Kyoto, Japan) with a flow-through cell (25 μl). Data acquisition and control of the FIA system were carried out with a software program written in LabView 6.1 (National Instruments Co., TX, USA). Unless otherwise stated, the FIA system was operated under the reference operating conditions, which were summarized in Table 1.

2.4. Biotechnological processes

Succinic acid is produced in a fermentation process as end product or can be used as precursor for the formation of secondary metabolic product such as 5-aminolevulinic acid (ALA) [16]. In this work, recombinant *E. coli* BL21(DE3)pLysS harboring the plasmid pFLS45 which employs succinic acid and glycine as precursors and produces ALA, was immobilized in calcium alginate gels and cultivated in a bioreactor with working volume of 2.5 l. The bioreactor was aerated only with 2 vvm air. The fermentation medium consists of 2 g l^{-1} glucose, 0.1 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g l^{-1} NH_4Cl, 0.2 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} KH_2PO_4, 20 mM glycine, 2.5 g l^{-1} succinic acid, 4 ml l^{-1} of trace element stock solution, 4 ml l^{-1} of vitamin stock solution and 75 mg l^{-1} ampicillin (trisodium salt) [17]. The pH value of medium is controlled to 6.2 by using 5 N NaOH solution. The cell free medium separated by using 0.2 μm polypropylene microfiltration tubular sampling module (ABS Co., Puchheim, Germany) was used to monitor the concentrations of succinic acid on-line by the FIA system developed in this work [15]. Before applying the FIA system to a real biological process, the concentration of succinic acid in a continuously stirred reactor was also on-line monitored by the FIA system.

3. Results and discussion

3.1. Characteristics of the immobilized ICL/ICDH-based FIA system

In the immobilized ICL/ICDH reactor, succinic acid is converted to 2-oxoglutarate via isocitrate in the presence of glyoxylate and $NADP^+$. Tsukatani and Matsumoto have found the optimal concentration of glyoxylate (10 mM) and $NADP^+$ (1 mM) for the determination of succinic acid by a FIA system [13]. However, they have used expensive HEPES buffer as carrier solution for the FIA system. So, in order to find a cheap, but efficient carrier buffer solution for the FIA system we have tested different kinds of buffer solutions used in other FIA systems [18]. Among these investigated buffer solutions, the sodium phosphate buffer solution showed high signal peaks and no salts were formed in the buffer solution, when other components like cysteine and glyoxylate were added and sample solution was introduced into the immobilized enzyme reactor. Therefore, we used the sodium phosphate buffer solution to study the characteristics of the FIA system with an immobilized ICL/ICDH reactor.

3.1.1. Stability

The success of the FIA system to monitor the concentration of succinic acid is dependent upon the stability of ICL and ICDH immobilized onto epoxy polymer carrier. To investigate the operational stability of the immobilized ICL/ICDH reactor, 1.0 g l^{-1} succinic acid was measured by the FIA system under the reference operating conditions shown in Table 1. The peak heights of the sample were kept almost constant, i.e. with about 94% of the initial peak height after successive injection of samples for 24 h (data not shown). The immobilized ICL/ICDH reactor was stored at 4 °C, when not in use. After storage in a refrigerator at 4 °C for 30 days the immobilized ICL/ICDH reactor kept its activity over 95% of the initial value.

3.1.2. Effects of pH and temperature

ICL and ICDH have normally their optimal pH values at 6.8 and 7.0, respectively. The ICL/ICDH immobilized on aminopropyl-controlled pore glass [13] had also the maximum activity at pH 7.0. However, in our previous studies some enzymes have shifted their maximum activity in the alkaline pH range after their immobilization on VA-epoxy support [14,15]. So, to investigate the effect of pH values on the activity of the immobilized ICL and ICDH, the pH values of the carrier buffer solution have been varied in the range of 6.0–10.5 by adding 5 M HCl and 5 M NaOH solution. The maximum peak height was obtained in the carrier solution of pH 9.0, and the peak height at pH 7.0 decreased to 85% of the peak height at pH 9.0. The result showed that

Table 2
Effects of certain components added to the carrier buffer solution on the peak height of 2.0 g l^{-1} succinic acid

Components added to carrier buffer solution	Relative peak height (%)
No	100
3.4 mM EDTA	101
1 mM IMD	98
5 mM Na-azide	97
10 mM Na-azide	96
0.1 mM DTT	96
1 g l^{-1} SCAD	101
2 g l^{-1} SCAD	104
3 g l^{-1} SCAD	105
0.5 g l^{-1} Triton X-100	105
2 g l^{-1} Urea	102

the optimum pH value of the immobilized ICL and ICDH can be varied according to carrier supports.

The effect of reaction temperature on the activity of the immobilized ICL and ICDH was investigated. The peak heights increased with increasing temperatures within the range of 22–35 °C. But at higher temperature, it was difficult to perform the experiments due to small bubbles in the tubing and within the cartridge.

3.1.3. Effects of components added to carrier buffer solution

ICL and ICDH can be activated or inhibited by certain components added to the carrier buffer solution. Imidazole (IMD), DL-dithiothreitol (DTT), ethylendiamine tetraacetic acid (EDTA), semicarbazide (SCAD), Triton X-100 and sodium azide (Na-azide) have been often dissolved in the carrier solution of FIA system in order to increase the activity of the immobilized enzymes as well as the lifetime of the carrier buffer solution [15]. The effects of these components added to the carrier buffer solution on the peak heights were shown in Table 2, when 2 g l^{-1} succinic acid was injected as sample. Under the concentrations here investigated there was no significant enhancement or inhibition to the enzymic reaction, that one can add 1 mM IMD or 10 mM Na-azide to the carrier buffer solution in order to enhance the lifetime of the buffer solution. A 3 g l^{-1} SCAD can be also introduced into the buffer solution as an activator for the production of 2-oxaloglutarate and NADPH.

3.1.4. Effects of $NADP^+$

There are a few kinds of ICDH, which are specific to either NAD^+ or $NADP^+$. For our work, the $NADP^+$-specific ICDH has been rather used than the NAD^+-specific ICDH, because the latter may change the conformation of the enzyme after binding some metabolites such as citrate to a unique site [19]. The effects of flow rates and concentrations of the $NADP^+$ solution on the conversion rate of succinic acid in the immobilized ICL/ICDH reactor were investigated using the reference operating conditions in Table 1. As shown in Fig. 2, there was little change in peak heights

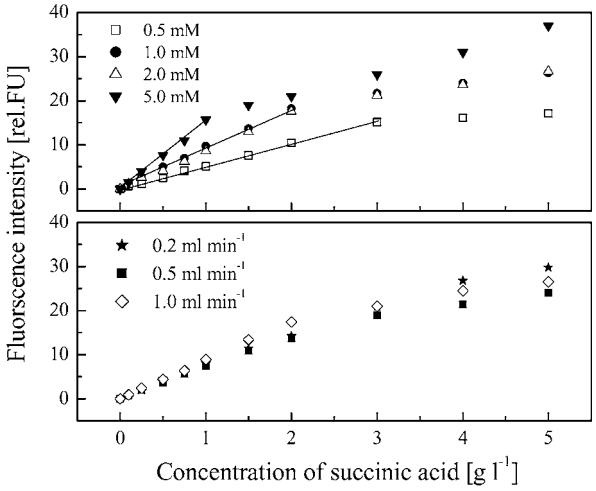

Fig. 2. Effects of flow rates and concentrations of the $NADP^+$ solution on the peak heights.

when increasing the flow rates of the $NADP^+$ solution in the range of 0.2–1.0 ml min^{-1}, as the NAD^+ solution in the FIA system for fumaric acid [15]. The conversion rate of succinic acid in the enzyme reactor also depends on the concentration of the $NADP^+$ solution as well as its quantity introduced. When the flow rate of the $NADP^+$ solution was kept constant at 1 ml min^{-1}, the peak heights increased with increasing concentration of the $NADP^+$ solution in the range of 0.5–5.0 mM, and the linear concentration ranges were varied, i.e. 0–3.0 g l^{-1} for 0.5 mM $NADP^+$ solution (r^2 = 0.999), 0–2.0 g l^{-1} for 1.0 and 2.0 mM $NADP^+$ solution (r^2 = 0.999) and 0–1.0 g l^{-1} for 5 mM $NADP^+$ solution (r^2 = 0.999). The optimal molar concentration of the $NADP^+$ solution for the FIA system should be selected under consideration of linear correlation, analysis cost and also sensitivity, i.e. peak height regarding to the concentration of succinic acid.

3.1.5. Effects of carrier flow rates

The effects of the flow rates of carrier buffer solution on the activity of the immobilized ICL and ICDH are

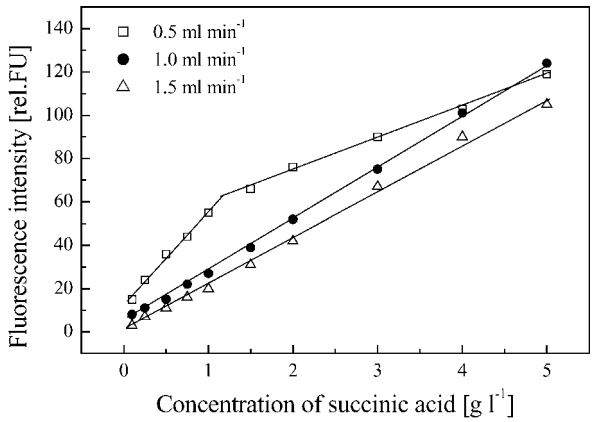

Fig. 3. Effects of flow rates of the carrier solution on the peak heights.

shown in Fig. 3, when the FIA system was operated under the conditions shown in Table 1. The conversion degree of succinic acid increased with decreasing flow rates of the carrier solution. With a flow rate of 0.5 ml min^{-1}, a linear calibration curve was obtained in the concentration range of 0.25–1.0 g l^{-1} (r^2 = 0.998) and 1.5–5.0 g l^{-1} (r^2 = 0.999) of succinic acid, while a linear calibration range was obtained in the range of 0.1–5.0 g l^{-1} (r^2 = 0.999) with 1.0 ml min^{-1} and in the range of 0.1–5.0 g l^{-1} (r^2 = 0.998) with 1.5 ml min^{-1} of carrier flow rates, respectively. Higher flow rate of the carrier solution would shift the linear calibration range to higher concentration range of succinic acid due to some mass transfer effects on the immobilized ICL and ICDH cartridge.

3.1.6. Effects of sample injection volume

The sample injection volume influences the conversion of succinic acid to 2-oxaloglutarate catalyzed by the two immobilized enzymes. The conversion rate, i.e. peak height of the FIA system was investigated in the sample injection range of 75–250 μl. The peak heights increased with increasing sample injection volumes. But, the choice of an optimal sample injection volume should be made as a compromise between sensitivity (peak height) and sample output rate (rapidity). Therefore, 125 μl of sample injection volume has been chosen in this work and used to monitor the concentration of succinic acid on-line.

3.1.7. Interferences

In biotechnological processes, some compounds are consumed and/or produced, that they are included in the sample and injected into the FIA system. These may cause activation or inhibition to the enzymatic reaction. In Table 3, the effects of some nutrient salts, substrates and metabolites on the enzymatic reaction are studied. The kinds and concentrations of nutrient salts and substrates investigated here are based on the maximum amounts added to the culture medium at the beginning of some fermentation processes [2,17], while the high concentrations of metabolites are taken from other cultivation processes [3]. The peak heights of 2.0 g l^{-1} succinic acid with some nutrient salts, substrates and metabolites added showed significant enhancement or reduction in comparison to the peak height of the 2.0 g l^{-1} succinic acid without additional components. The presence of 3.0 g l^{-1} acetate and 0.1 g l^{-1} $FeSO_4$ in a sample solution caused a reduction in the peak height to about 13%. High concentrations of certain compounds, e.g. 10 g l^{-1} lactose, 1.0 g l^{-1} citrate, 3.0 g l^{-1} malate and oxaloacetate played a role as activators for the enzymic reaction and increased the peak heights more than 15%. To eliminate the effects of the activation and inhibition of the compounds on the enzymic reaction, samples can be diluted adequately or standard solutions containing these compounds can be prepared and used to make the calibration curve. When all components, i.e. nutrients salts, substrates and metabolites studied in Table 3 had been added to the sample solution (2.0 g l^{-1} succinic acid), respectively, there was no significant inhibitory or activating effects on the activity of the immobilized ICL and ICDH.

Table 3
Effects of nutrient salts, substrates and metabolites contained in samples on the peak height of 2.0 g l^{-1} succinic acid

	Relative peak height (%)
(a) Nutrient salts added to sample	
No	100
1.0 g l^{-1} $CaCl_2$	110
0.1 g l^{-1} $FeSO_4$	87
1.0 g l^{-1} K_2HPO_4	97
1.0 g l^{-1} $MgCl_2$	98
1.0 g l^{-1} NaCl	97
1.0 g l^{-1} $(NH_4)_2SO_4$	100
All nutrient salts	94
(b) Substrates added to sample	
No	100
10 g l^{-1} L-Arabinose	98
10 g l^{-1} D-Fructose	96
10 g l^{-1} D-Glucose	101
10 g l^{-1} D-Inositol	91
10 g l^{-1} Lactose	101
10 g l^{-1} Maltose	113
10 g l^{-1} Mannose	95
10 g l^{-1} Sucrose	98
All substrates	98
(c) Metabolites added to sample	
No	100
3 g l^{-1} Acetate	87
1 g l^{-1} Citrate	116
5 g l^{-1} Fumarate	90
3 g l^{-1} Malate	124
3 g l^{-1} Oxaloacetate	127
2 g l^{-1} Pyruvate	107
All metabolites	99

3.2. On-line monitoring of biotechnological processes

To test the performance of the FIA system for on-line monitoring of the succinic acid concentration, a continuously stirred tank reactor was employed with 1.0 ml min^{-1} influent and effluent rate. In Fig. 4, 20 g l^{-1} succinic acid was first pumped into a 200 ml working volume reactor containing distilled water. After 5.5 h, the influent was exchanged and distilled water was pumped into the reactor. The effluents from the reactor were monitored on-line with the FIA system under the reference operating conditions specified in Table 1. After 3.0 h, the samples of the FIA system were diluted to 1:2. The on-line monitored data were compared with the off-line data measured by a HPLC system (Shimadzu SPD-10Avp UV–vis detector at 210 nm, μBondapak C_{18} column at 20 °C, a carrier solution of 0.008 N H_2SO_4 with 1.0 ml min^{-1}, sample injection volume of 20 μl). The linear calibration curve for standard solution of succinic acid by the HPLC had a correlation coefficient of r^2 = 0.9995 (seven data points) over the range 0.1–0.5 g l^{-1}. Comparison of the HPLC data with FIA data showed good trend conformity.

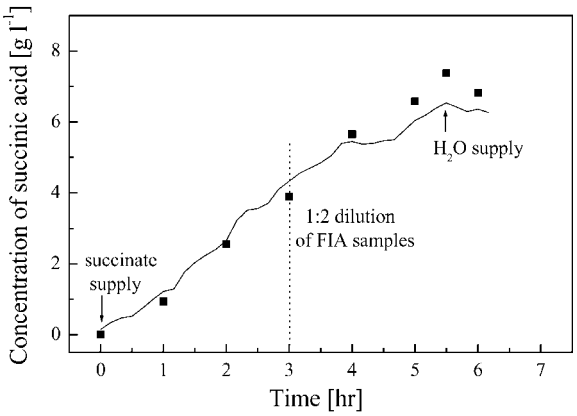

Fig. 4. On-line monitoring of the concentrations of succinic acid in a continuously stirred tank reactor. Symbols are off-line data measured by the HPLC system.

During the fermentation of recombinant *E. coli* BL21 (DE3)pLysS harboring the plasmid pFLS45 immobilized in calcium alginate gels, the concentration of succinic acid was monitored on-line, as shown in Fig. 5. Off-line samples were taken from the waste behind the injector. A blank cartridge filled with epoxy carrier supports was also integrated into the FIA system in order to study the change of the fluorescence intensity at 340 nm (ex)/440 nm (em) in the cell free culture medium during the fermentation. The recombinant *E. coli* began to consume succinic acid in the beginning of the fermentation and after 2 h low concentration of succinic acid was found in the culture medium, so that 70 ml concentrated solution of succinic acid (100 g l^{-1}) was added into the bioreactor via syringe. However, due to dead volume (ca. 5 ml) in the inner column of the membrane sampling module and slow liquid mixing in the reactor, the concentration of succinic acid increased slowly with time (Fig. 5). After 5.8 h, the concentrations of succinic acid begun to decrease. The samples of the FIA system were diluted to 1:2 after 4 h. Some metabolites like citric acid would be produced during the fermentation and may interfere the monitoring of succinic acid by the FIA system [19]. Low concentrations of organic acids like citric acid (<0.5 g l^{-1}, measured with HPLC) were produced during the fermentation, so it was not necessary to dilute the samples or to use standard solutions containing some organic acids. During the fermentation, the peak heights of samples introduced into the blank cartridge did not also change significantly. The reason is, the cell free samples separated by the membrane sampling module contained little fluorophors (e.g. NAD(P)H) at 340 nm (ex)/440 nm (em). Therefore, relative good analogy between the on-line FIA data and off-line HPLC data proved the validation of the FIA system developed in this work.

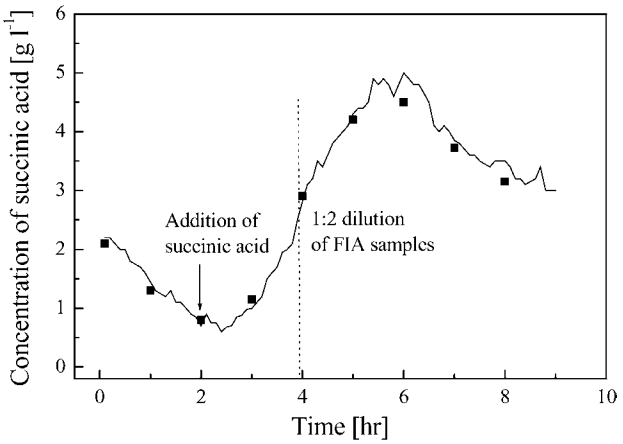

Fig. 5. On-line monitoring of the succinic acid concentration during a fermentation of recombinant *E. coli* immobilized in calcium alginate gels. Symbols are off-line data measured by the HPLC system.

4. Conclusion

Two enzymes, ICL and ICDH, were immobilized on VA-Epoxy Biosynth E3-carrier and characterized for the development of a FIA systems for monitoring of the succinic acid concentrations in biotechnological processes. The immobilized ICL and ICDH reactor has long operational lifetime (stable at least for 24 h) and storage stability (stable up to 1 month at 4 °C). Optimum pH value of the immobilized ICL and ICDH reactor was shifted in the alkaline range like other enzymes immobilized on epoxy carriers. The activity of the immobilized ICL and ICDH was not largely influenced by some components such as IMD (1 mM), Na-azide (10 mM) and SCAD (2 g l^{-1}) added to the carrier buffer solution. But, certain compounds, e.g. maltose (10 g l^{-1}), citrate (3 g l^{-1}), malate (3 g l^{-1}) and oxaloacetate (3 g l^{-1}) in sample solution showed activating effect of more than 10% on the enzyme activity, while acetate (3 g l^{-1}) and $FeSO_4$ (3 g l^{-1}) had significant inhibitory effects on the enzyme activity. Therefore, sample matrix effects have to be taken into account for the on-line monitoring of the succinic acid concentrations in bioprocesses. The on-line monitoring data of the succinic acid concentrations in a fermentation of immobilized recombinant *E. coli* showed a high degree of correspondence to the off-line data measured by the HPLC system. With its good sensitivity and reliability, the FIA system developed in this work can further be applied to monitor succinic acid.

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