Continuous Production of L-Alanine with NADH Regeneration by a Nanofiltration Membrane Reactor

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A conjugated enzyme system, alanine dehydrogenase (AIDH) for stereospecific reduction of pyruvate to L-alanine and glucose dehydrogenase (GDH) for regeneration of NADH, were coimmobilized in a nanofiltration membrane bioreactor (NFMBR) for the continuous production of L-alanine from pyruvate with NADH regeneration. Since pyruvate was proved to be unstable at neutral pH, it was kept under acidic conditions and supplied to NFMBR separately from the other substrates. As 0.2 mM pyruvate in HCl solution (pH 4), 10 mM NAD, 0.2 mM glucose, and 0.2 mM NH4Cl in 0.5 M Tris buffer (pH 8) were continuously supplied to NFMBR with immobilized AIDH (100 U/ml) and GDH (140 U/ml) at the retention time of 80 min, the maximum conversion, reactor productivity, and NAD regeneration number were 100%, 320 g/liter/d, and 20,000, respectively. To avoid the effect of pyruvate instability, a consecutive reaction system, lactate dehydrogenase (L-LDH) and AIDH, was also used. In this system, the L-LDH provides pyruvate, the substrate for the AIDH reaction, from L-lactate regenerating NADH simultaneously, so the pyruvate could be consumed as soon as it was produced. As 0.2 mM L-lactate, 10 mM NAD, 0.2 mM NH4Cl in 0.5 M Tris buffer (pH 8) were continuously supplied to NFMBR with immobilized L-LDH (100 U/ml) and AIDH (100 U/ml) at the retention time of 160 min, the maximum conversion, reactor productivity, and the NAD regeneration number were 100%, 160 g/liter/d, and 20,000, respectively.

Key words: nanofiltration membrane bioreactor; alanine dehydrogenase; L-lactate dehydrogenase; glucose dehydrogenase; NADH regeneration

Although L-alanine is commercially produced by decarboxylation of L-aspartic acid catalyzed by immobilized L-aspartate-β-decarboxylase,1–3 reductive amination of pyruvate catalyzed by L-alanine dehydrogenase can be an alternative way to produce L-alanine.4–6 This method to produce an L-amino acid from the corresponding keto-acid is also applicable to produce various amino acids such as L-leucine,7,8 L-valine,8 L-isoleucine,8 and L-phenylalanine.9 For the continuous production of L-amino acids by this method, however, the reactor system is inevitably provided with a coenzyme (NADH) regeneration system because of the cost of the coenzyme.10 To this end, chemically modified macromolecule-bound NAD11,12 has been extensively studied to immobilize the coenzyme in a membrane reactor12 or in microcapsules.8

In our previous paper,14 we proposed a nanofiltration membrane reactor (NFMBR) in which dissociable coenzyme such as NAD can be partly entrapped and immobilized along with the conjugated enzyme system for the main reaction and the regeneration reaction of coenzyme. In this method, native coenzyme can be used instead of chemically modified coenzyme. This makes the system much simpler and the process less expensive because the cost of coenzyme is a limiting factor in this type of process.5,10

In this paper, NFMBR was used to produce L-alanine continuously from pyruvate or L-lactate with NADH regeneration by the conjugated enzyme system of alanine dehydrogenase (AIDH) and glucose dehydrogenase (GDH) or that of AIDH and L-lactate dehydrogenase (L-LDH).

Materials and Methods

Materials. Alanine dehydrogenase (=AIDH, EC 1.4.1.1, Bacillus subtilis), and L-lactate dehydrogenase (=L-LDH, EC 1.1.1.27, Bovine Heart) were purchased from Sigma Chemical Co., U.S.A. Glucose dehydrogenase (=GDH, EC 1.1.1.47, Bacillus sp.) was kindly supplied by Amano Pharmaceutical Co. Ltd. NAD was purchased from Boehringer Mannheim, Germany. Nanofiltration membrane UTC-20 was kindly supplied by Toray Industries Inc. All other chemicals used were of reagent grade.

Enzyme unit definition. The enzyme unit definition was a nominal unit volume guaranteed on the bottle decomposing 1.0 µmol of substrate per min. This definition was for deamination of L-alanine at pH 10 at 25°C for AIDH and for reduction of pyruvate at pH 7.5 at 37°C for L-LDH. The actual activity of AIDH for deamination at pH 8 at 25°C was measured to be 328% of the nominal value and that of L-LDH for oxidation of L-lactate at pH 8 at 25°C was 8.6% of the nominal value. The activity of GDH to oxidize glucose at pH 8 at 25°C was also measured to be 128% of the nominal value.

Batchwise reaction

1. AIDH/GDH conjugated enzyme system. The reaction scheme is shown in Scheme 1. Pyruvate (0.1 µl), glucose (0.2 µl), NH4Cl (0.2 µl), and NAD (0.1 nm) were mixed together (5 ml in volume) in 0.5 M Tris buffer (pH 8). AIDH and GDH (35 U/ml) were added to start the reaction at 25°C.

2. L-LDH/AIDH consecutive enzyme system. The reaction scheme is

Scheme 1. Conjugated Enzyme System of AIDH and GDH to Produce L-Alanine.

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shown in Scheme 2. L-Lactate (0.1 m), NH₄Cl (0.1 m), and NAD (0.1 mM) were mixed together (5 ml in volume) in 0.5 M Tris buffer (pH 8). AIDH (10 U/ml) and L-LDH (10 U/ml) were added to start the reaction at 25°C.

Continuous production of l-alanine

1. Single channel NFMBR with AIDH/GDH conjugated enzyme system. GDH (140 U/ml) and AIDH (33 or 100 U/ml) were coimmobilized in NFMBR (2 ml in volume, no stirring), and the substrate solution containing 0.2 M pyruvate, 0.2 M NH₄Cl, 0.2 M glucose, and 10 mM NAD in 0.5 M Tris buffer (pH 8) was continuously supplied via a degasser (Jasco, DG-900-50) with a HPLC pump (PU-980, Jasco Co.). The NFMBR was immersed in a water bath kept at 25°C. The effluent from the NFMBR was collected by a fraction collector (Bio-Rad, 2111) and analyzed for l-alanine.

2. Double channel NFMBR with AIDH/GDH conjugated enzyme system. GDH and AIDH were coimmobilized in NFMBR. The substrate solution containing L-lactate, NH₄Cl, and a low concentration of NAD in 0.5 M Tris buffer (pH 8) was supplied continuously via a degasser by an HPLC pump to NFMBR.

Fig. 1. Batch Reaction to Produce L-Alanine by the Conjugated Enzyme System of AIDH and GDH.

The reaction mixture contained 0.1 mM NAD, 0.2 mM glucose, 0.1 mM pyruvate, 0.2 M NH₄Cl, and 35 U/ml GDH in 0.5 M Tris–HCl buffer (pH 8) along with AIDH (– – – – – – – – – – , 10.0 U/ml; – – – – – – – – – – , 0.3 U/ml; – – – – – – – – – – , 0.1 U/ml).

Fig. 2. Continuous Production of L-Alanine by Single Channel NFMBR Immobilized Conjugated Enzyme of AIDH and GDH.

The reaction was done by the continuous feeding of 0.1 mM NAD, 0.2 mM glucose, 0.2 mM pyruvate, and 0.2 M NH₄Cl in 0.5 M Tris–HCl buffer (pH 8) at a retention time of 80 min to NFMBR with immobilized GDH (140 U/ml) and AIDH (– – – – – – – – – – , 100 U/ml; – – – – – – – – – – , 33 U/ml).

Results and Discussion

Batchwise reaction to produce l-alanine with conjugated enzymes of AIDH and GDH

The results of batchwise reaction in Scheme 1 are shown in Fig. 1. The concentrations of AIDH and GDH affected the reaction rate but the maximum conversion at the appropriate conditions was almost 100%, showing that the conjugated enzyme system GDH/AIDH works well to produce L-alanine from pyruvate with NAD regeneration. In this case, the regeneration number of NAD was 1000.

Continuous production of L-alanine by single-channel NFMBR with AIDH/GDH conjugated enzymes

Figure 2 shows the result of continuous production of L-alanine by a single-channel NFMBR with immobiobilized AIDH/GDH. With 33 U/ml AIDH and 100 U/ml GDH immobilized in NFMBR, the maximum conversion was 85%, the NAD regeneration number was 16,000, and the reactor productivity at the peak was 275 g/liter/d. The half-life of NFMBR, however, was only 10 h. When the AIDH concentration was raised to 100 U/ml, the maximum conversion, NAD regeneration number, and productivity became 95%, 20,000, and 323 g/liter/d, respectively. The half-life on NFMBR was improved a little but it was still only 30 h. These results show that the conjugated enzyme system of AIDH/GDH immobilized in NFMBR can produce L-alanine continuously from pyruvate with simultaneous NADH regeneration but the operational stability of NFMBR was quite short. To circumvent this problem the stability of the substrate, pyruvate, was investigated.

Stability of pyruvate

At a neutral pH, pyruvate can be decomposed, releasing carbon dioxide before entering NFMBR because the reactor system have a large dead volume in the degasser. The expected residence time of the substrates in the reservoir and the degasser is more than 10 h. Figure 3 shows the stability of pyruvate at a neutral pH and an acidic pH. The pyruvate was unstable at pH 8 in 0.5 M Tris buffer while it was stable at pH 4. Pyruvate concentration was reduced to 50% at pH 8 in 15 h. Therefore, pyruvate was kept at pH 4.
Continuous production of L-alanine by double-channel NFMBR with AIDH/GDH conjugated system

Figure 4 shows the results of the double-channel NFMBR in which pyruvate was supplied to the reactor separately.

Compared with the single-channel case (Fig. 2), the operational stability of the reactor was much improved and an almost 100% conversion ratio was obtained with the immobilized AIDH concentration at 100 U/ml.

Table I summarizes the results for continuous production of L-alanine from pyruvate by NFMBR at various conditions. A change in the immobilized enzyme (AIDH) concentration from 33 to 100 U/ml did not affect the reactor performance much. The enzyme concentration at 33 U/ml seems almost enough for the high conversion under these experimental conditions. The increase in the enzyme concentration probably affects the operational stability of NFMBR although the complete test of the operational stability was not carried out because it is so time-consuming.

As for the effect of coenzyme (NAD) in the feed, 10 μM seems likely to be enough, because the NAD in NFMBR is concentrated through the rejection of the nanofiltration membrane (UTC-20). As the rejection ratio of UTC-20 to NAD in 0.5 M Tris buffer is 0.96, the expected NAD concentration in the NFMBR from this rejection ratio is 250 μM. The actual NAD concentration in NFMBR, however, will be higher than this because of the concentration polarization on the nanofiltration membrane, which would not have worked in the measurement of the rejection ratio of UTC-20 to NAD at a very low concentration. Therefore, the NAD concentration in NFMBR would be higher than the K_m of GDH (100 μM) and the regenerated NADH concentration would be higher than the K_m of AIDH (23 μM).

To increase the reactor productivity, the residence time was changed from 80 to 40 min. The results, however, were not as expected and the reactor productivity was not much improved. So, the substrate concentration was increased from 0.2 to 0.4 M but this inhibited the enzyme reaction.

The best result from Table I was obtained with the AIDH concentration at 100 U/ml, NAD concentration at 10 μM, substrate concentration at 0.2 M and the retention time of 80 min. In this condition, the reactor productivity was 320 g/liter/d with an NAD regeneration number of 20,000, which allows reduction of the NAD cost to something.

Table I. Summary of NFMBR for Continuous Production of L-Alanine with Conjugated Enzyme System of AIDH and GDH

<table>
<thead>
<tr>
<th>[GDH] (U/ml)</th>
<th>[AIDH] (U/ml)</th>
<th>[NAD] (μM)</th>
<th>[Pyruvate] (μM)</th>
<th>t_R (min)</th>
<th>Conv. (%)</th>
<th>N_R</th>
<th>Productivity (g/liter/d)</th>
<th>Half-life (h)</th>
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</thead>
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<tr>
<td>(a) Single channel NFMBR</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>33</td>
<td>10</td>
<td>0.2</td>
<td>80</td>
<td>0.85</td>
<td>17,000</td>
<td>270</td>
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</tr>
<tr>
<td>140</td>
<td>100</td>
<td>10</td>
<td>0.2</td>
<td>80</td>
<td>1.00</td>
<td>20,000</td>
<td>320</td>
<td>30</td>
</tr>
<tr>
<td>(b) Double channel NFMBR</td>
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<td></td>
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<tr>
<td>140</td>
<td>33</td>
<td>10</td>
<td>0.2</td>
<td>80</td>
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<td>16,000</td>
<td>260</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>140</td>
<td>100</td>
<td>10</td>
<td>0.2</td>
<td>80</td>
<td>1.00</td>
<td>20,000</td>
<td>320</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>150</td>
<td>90</td>
<td>1</td>
<td>0.2</td>
<td>80</td>
<td>0.88</td>
<td>16,000</td>
<td>26</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>150</td>
<td>50</td>
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<td>0.2</td>
<td>80</td>
<td>0.80</td>
<td>45,000</td>
<td>257</td>
<td>&gt; 150</td>
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<tr>
<td>150</td>
<td>90</td>
<td>10</td>
<td>0.2</td>
<td>80</td>
<td>1.00</td>
<td>20,000</td>
<td>320</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>140</td>
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<td>35</td>
<td>0.2</td>
<td>40</td>
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<td>3,140</td>
<td>353</td>
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<td>10</td>
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<td>0.45</td>
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<td>—</td>
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<td>80</td>
<td>0.35</td>
<td>14,000</td>
<td>225</td>
<td>—</td>
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</table>

* Concentrations of NH_4Cl and glucose were the same as that of pyruvate.
* t_R: retention time.
* Conv.: conversion rate at the peak.
* Productivity: productivity at the peak.
almost negligible, 1/20,000 in this process.

**Batchwise reaction to produce l-alanine with consecutive enzyme of l-LDH and AIDH**

To avoid the effects of the instability of pyruvate, the pyruvate produced can be immediately consumed in a consecutive reaction. For this purpose, a conjugated enzyme system of l-LDH/AIDH is available. In this case, the starting material is l-lactate, which is oxidized to pyruvate consuming NAD catalyzed by l-LDH. The product, pyruvate, then is reduced to l-alanine by AIDH as in Scheme 2.

Figure 5 shows the results for the batchwise conjugated reaction of l-LDH and AIDH in a beaker. The reaction proceeded well and 100% conversion of l-lactate to l-alanine was obtained in 500 min under these experimental conditions. This shows that the overall reaction is favorable to produce l-alanine although the first stage l-LDH reaction is not thermodynamically favorable to produce pyruvate. In this case, the regeneration number of NAD was 1000.

**Continuous production of l-alanine with l-LDH/AIDH consecutive enzyme system**

Figure 6 shows the continuous production of l-alanine from l-lactate by NFMBR with immobilized l-LDH (100 U/ml) and AIDH (100 U/ml). At the residence time of 160 min, the maximal conversion of l-lactate to l-alanine was almost 100%. The operational stability of NFMBR was very good and no apparent decrease in the productivity was observed at 120 h from the start of the reaction, showing that the effect of the pyruvate instability could be effectively avoided.

Table II summarizes the results to produce l-alanine continuously from l-lactate by NFMBR with the l-LDH/AIDH system. The immobilized enzyme concentration was systematically changed from 50 to 200 U/ml for l-LDH and from 50 to 150 U/ml for AIDH but no substantial effect of enzyme concentration was observed on the reactor behavior of NFMBR probably because the reaction in NFMBR was equilibrated at these conditions. The conversion, however, was restricted to as low as 60% at most. The accumulation of some materials such as l-alanine in the reactor might have affected the reaction equilibrium. This effect could be avoided by decreasing the substrate concentration from 0.2 to 0.1 M or by increasing the residence time from 80 to 160 min. In the latter case, the accumulation of materials in NFMBR could have been avoided because of the lower rejection by the nanofiltration membrane at the lower flux through less concentration polarization.

At the residence time of 160 min, however, the conversion decreased from 100 to 45% upon increasing the substrate

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**Table II. Summary of NFMBR for Continuous Production of l-Alanine with Consecutive Enzyme System of l-LDH and AIDH**

<table>
<thead>
<tr>
<th>[l-LDH] (U/ml)</th>
<th>[AIDH] (U/ml)</th>
<th>[NAD] (μM)</th>
<th>[l-Lactate] (mM)</th>
<th>Conv (%)</th>
<th>N&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Productivity&lt;sup&gt;d&lt;/sup&gt; (g/liter/d)</th>
<th>Half-life (h)</th>
</tr>
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<tbody>
<tr>
<td>50</td>
<td>100</td>
<td>10</td>
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<td>80</td>
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<td>10,000</td>
<td>160</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>10</td>
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<td>80</td>
<td>0.60</td>
<td>12,000</td>
<td>190</td>
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<tr>
<td>200</td>
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<td>10</td>
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<td>80</td>
<td>0.60</td>
<td>12,000</td>
<td>190</td>
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<td>80</td>
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<td>175</td>
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<td>10</td>
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<td>160</td>
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<tr>
<td>100</td>
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<td>10</td>
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<td>160</td>
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<td>144</td>
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<td>80</td>
<td>0.88</td>
<td>1,760</td>
<td>282</td>
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</table>

<sup>a</sup> Concentrations of NH₄Cl was the same as that of l-lactate.

<sup>b</sup> t<sub>r</sub>, retention time.

<sup>c</sup> Conversion ratio at the peak.

<sup>d</sup> Productivity of NFMBR at the peak.
concentration from 0.2 to 0.4 M probably because of the accumulation of an inhibitory material in NFMBR again. Another possible explanation of the lower conversion at a high lactate concentration is inhibitory effect of the l-lactate to AIDH reaction. The inhibition constant of l-lactate was measured to be 0.11 M in the competitive mechanism.

As for the effects of NAD concentration in the feed, it was increased from 10 to 100 \( \mu \text{M} \) at the residence time of 80 min. Then a slight increase in the conversion from 60 to 80% was observed.

With the conjugated system of l-LDH/AIDH in NFMBR, the best result was obtained with l-LDH and AIDH concentrations at 100 U/ml, NAD concentration at 10 \( \mu \text{M} \), l-lactate concentration at 0.2 M, and the retention time at 160 min. In this condition, the maximal conversion was 100%, the reactor productivity was 160 g/liter/d, and the NAD regeneration number was 20,000 at the peak. These figures suggest the practical applicability of the present system.

In this system, the starting material was l-lactate, which is very expensive. Practically, however, \( \alpha \)-l-lactate will be available as a starting material. In this case, \( \beta \)-LDH may be used together with l-LDH to use \( \alpha \)-lactate as well as l-lactate. In the literature, use of a consecutive conjugated enzyme system of malic enzyme and AIDH was also proposed to produce l-alanine. In this process, the starting material is l-malate, which may be an alternative with economical feasibility.

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