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0273-2289/97/6701-02—0059$11.00

Study on Glucose Oxidase Fermentation Coupled with Membrane Dialysis

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Received September 11, 1996; Accepted December 17, 1996

ABSTRACT

The repression of glucose oxidase (GOD) biosynthesis by catabolites during fermentation can be alleviated through membrane dialysis fermentation (MDF). The results show that the volumetric enzyme productivity of MDF was two times higher than that of the control (fermentation without dialysis), and its total enzyme activity was increased by 30–50%. The operation conditions of MDF, such as pore size of the membrane, initiating time for membrane dialysis, and volume of dialysate used, were optimized. The content of amino acids and organic acids in the fermentation broth and the dialysate in the reservoir were assessed by amino acid analyzer and ionic chromatography, respectively. The relationship among the contents of pyruvic acid, gluconic acid, and enzyme activity during fermentation was analyzed quantitatively. Furthermore, the effect of membrane dialysis technology applied to the low-yield strain was found to be more effective than that applied to the high-yield strain.

Index Entries: Glucose oxidase; fermentation; membrane dialysis; *Penicillium* sp.; catabolite repression.

INTRODUCTION

Generally, *Aspergillus* or *Penicillium* sp. are used as the producer strain for the production of glucose oxidase (EC 1.1.4.3) (GOD). Glucose is not only used as carbon source, but also an inducer during GOD fermentation, and its catabolite acts as a repressor as well *(1)*. It is important to alleviate glucose catabolite repression or end-product repression by means of culture breeding or recombinant DNA technology. However, the increase in GOD

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productivity through mutagenesis would be eventually and inevitably restricted. In addition, the enhancement of enzyme activity can also be made by improvement of fermentation equipment and condition *(2–5)*. In conventional batch culture, the exhaustion of nutrients and the accumulation of detrimental metabolites during fermentation are inevitable. It is feasible to overcome such problems through feeding of nutrients and membrane dialysis *(6–8)*. In this article, membrane dialysis was used to enhance the productivity of GOD, and its optimal conditions and the factors regulating GOD biosynthesis were studied through analysis of their contents in culture broth and dialysate.

MATERIALS AND METHODS

Microorganism and Culture Medium

The GOD producer strain used in this study was *Penicillium* sp., which was isolated from soil and has been subjected to mutagenesis and rational screening in our laboratory. It has been proven to be a high-yield extracellular GOD producer *(9)*. The composition of nutrient agar used for preservation was as follows (g/L): sucrose 30, $NaNO_3$3, $KH_2PO_4$1, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, and agar 20 in distilled water with natural pH. The medium for preculture consisted of (g/L): glucose 40, soybean meal 5, $NaNO_3$2, $KH_2PO_4$1, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, and $FeSO_4 \cdot 7H_2O$ 0.01 in tap water with pH unchecked. The medium for production containing (g/L): glucose 80, $NaNO_3$8, peptone 3, $KH_2PO_4$2, KCl 0.5, and $MgSO_4 \cdot 7H_2O$ 0.7 in tap water with pH 7.7.

The nutrient agar slant was incubated at 28°C for 6 d and preserved at 4–6°C. Precultures were grown in 250-mL Erlenmeyer flasks with a working volume of 30 mL on a rotary shaker under 220 rpm at 28°C for 48 h. GOD fermentation was carried out in a 2.6-L capacity bench-top jar fermenter under the following conditions: working volume 1.5 L, amount of inoculum 5% (v/v), temperature 28 ± 0.3°C, stirrer speed 500–800 rpm.

Membrane Dialysis Module

The membrane dialysis module used in this study is shown in Fig. 1. The membranes were made of acetyl cellulose with pore sizes ranging from 0.30–0.80 µm. The total effective area of the unit of dialysis module was ca. 115 cm^2.

Membrane Dialysis Fermentation (MDF) System

The fermentation system coupled with membrane dialysis module is shown in Fig. 2. Batch culture was carried out at first in the system. At certain time of fermentation, the peristaltic pump was started. The culture broth was recycled on one side of the membrane, while the dialysate was

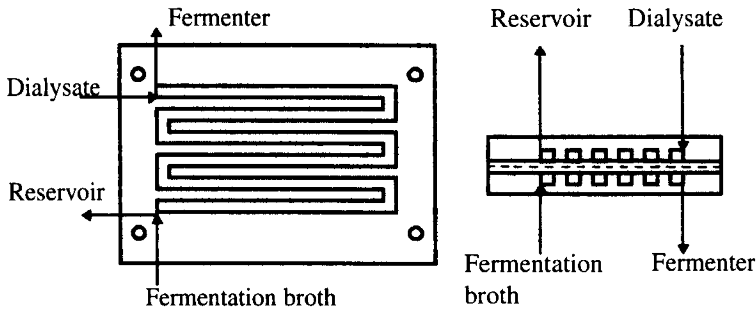

Fig. 1. Schematic diagram of the membrane dialysis module.

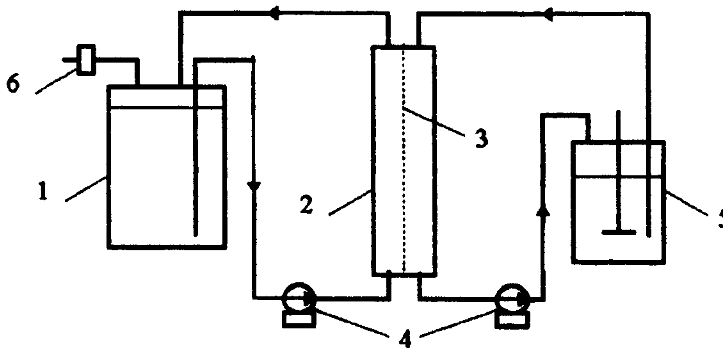

Fig. 2. Schematic diagram of membrane dialysis fermenter: 1. dialysate reservoir, 2. membrane dialysis module, 3. membrane, 4. peristaltic pump, 5. fermenter, 6. filter.

recycled on the other side of the membrane at a certain speed. The level of the culture broth in the fermenter was maintained by adjusting the flow rates of the fluids on both sides of the membrane, so that the metabolites and products from the culture broth could be diffused to the dialysate by concentration gradient, and the nutrient supplemented in the dialysate was able to diffuse to the culture broth as well. Otherwise stated, the pore size of the membrane was 0.65 μm; the recycle speeds of the culture broth and dialysate were set at 1700 and 720 mL/h, respectively, and the volume ratio of dialysate to culture broth was 2:1.

Analytic Procedures

Enzyme Assay

GOD activity was determined by monitoring the change in A_{500} owing to oxidation of *o*-dianisidine by horseradish peroxidase. In a cuvet, 2.4 mL of 0.21 m*M* *o*-dianisidine at pH 5.1, 0.05*M* acetate buffer, 0.5 mL of 10% (w/v) glucose solution, and 0.1 mL of horseradish peroxidase (1 U/mL) were added and equilibrated in a thermostat water bath at 35°C for 5 min.

The absorbance increase at 500 nm followed with time was immediately measured in Model 721 spectrophotometer, after 0.1 mL of sample had added and mixed thoroughly. GOD activity was evaluated from the rate of A_{500} increase by the following expression:

GOD activity (U/mL) = ΔA/min $\times$ total vol of reaction mixture/ (7.5* $\times$ vol of sample added) (1) *7.5 = molar extinction coefficient.

The GOD unit is defined as the amount of enzyme necessary for the oxidation of 1 µmol D-glucose to gluconic acid and H_2O_2/min at 35°C, pH 5.1.

Determination of Gluconic Acid

One mole of formaldehyde was generated when 1 mole of gluconic acid was oxidized by periodate. The excess periodic acid and HIO_3 were reduced by sulfurous acid solution until the yellow color had just apparently faded. The formaldehyde generated reacted with acetyl acetone and gave rise to a yellow color compound, diacetyl-3, 5-dimethyl-dihydropyridine, which was stable for 10 h, and the color developed could be measured by spectrophotometer.

Determination of Pyruvic Acid

Pyruvic acid could react with 2,4-dinitrophenylhydrazine to give rise to acetonedinitro-phenylhydrazone, the later compound would develop a brown color under strong alkaline condition, and its OD value could be measured at 520 nm.

Determination of Other State Variables

Dry cell weight was measured after 10 mL of broth were filtered, washed with demineralized water, and dried at 105°C until constant weight was gained. Glucose was determined as reduced sugar by dinitrosalicylic acid reagent *(10)*. NH_2-N was assayed by the formaldehyde method *(11)*. Dissolved oxygen and pH were monitored with Galvanic-type DO electrode, and combined glass and reference electrode, respectively.

RESULTS AND DISCUSSION

GOD Batch Fermentation (BF)

The time-courses of various state variables during GOD fermentation were depicted in Fig. 3. During the initial 24 h of fermentation, the consumption of glucose was rather slow. As the cell concentration increased, glucose consumption and GOD synthesis were accelerated, and pH declined to 3.0 at 16 h of fermentation. The enzyme volumetric productivity during 0–16 h was 0.37 U/mL · h; and raised to 2.42 U/mL · h between 16 and 36 h of fermentation. GOD activity reached its maximum, 54.3 U/mL, at 36 h after inoculation.

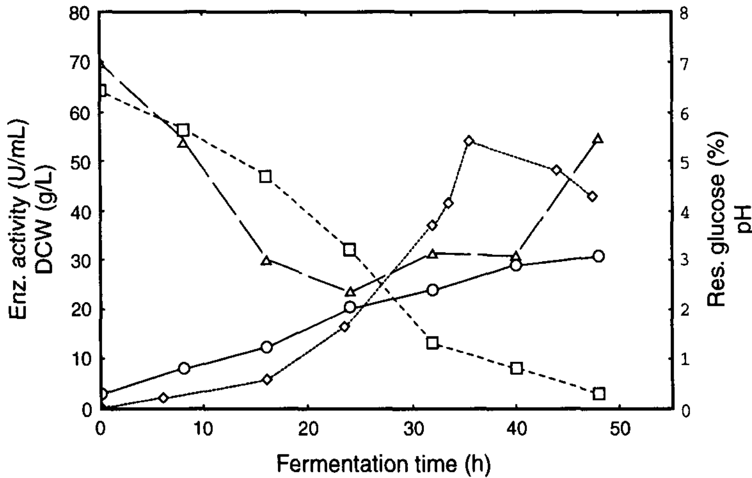

Fig. 3. Time-courses of various state variables during GOD batch fermentation Symbols: residual glucose (□), dry cell weight (○), pH (△), enzyme activity (◇).

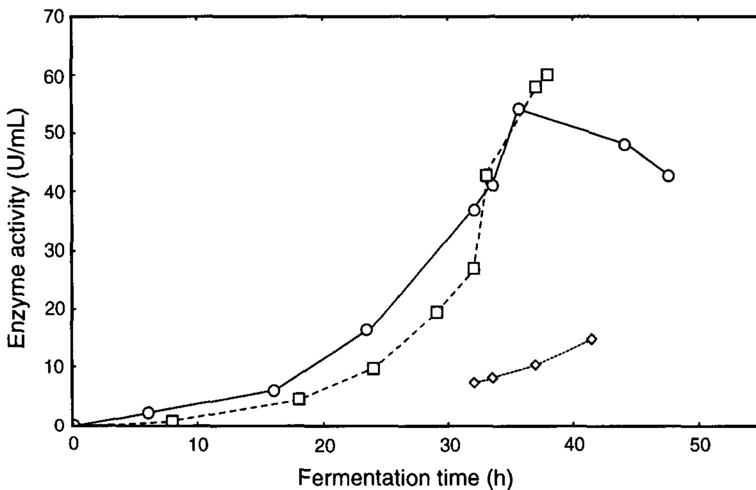

Fig. 4. Comparison of the profiles of enzyme activity among BF (○), MDF, (□) and dialysate (◇).

Characteristics of GOD Fermentation with Membrane Dialysis

The operation conditions for MDF at an early period were the same as BF. Cell recycle and membrane dialysis were started at certain times of fermentation. As shown in Fig. 4, although the enzyme activity at 32 h in MDF (26.8 U/mL) was lower than that in BF (37.2 U/mL), but after the membrane dialysis had started, the enzyme productivity in MDF was nearly two times as high as that in BF, and the enzyme productivity in MDF and BF was 5.58 (the enzyme activity in the dialysate was not included)

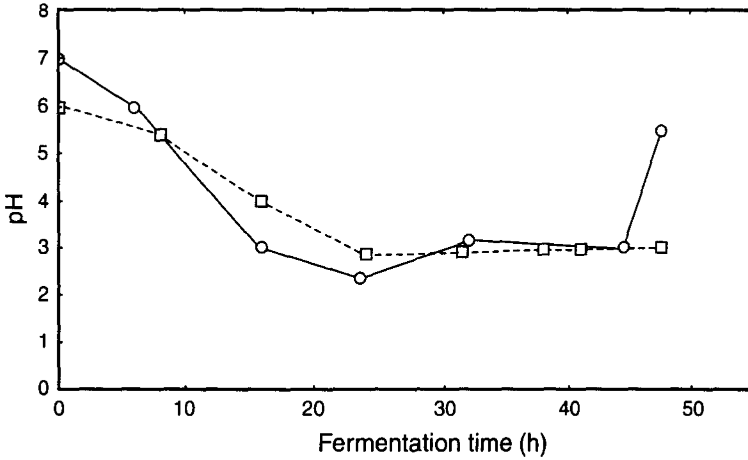

Fig. 5. Comparison of pH profiles between MDF (□) and BF (○).

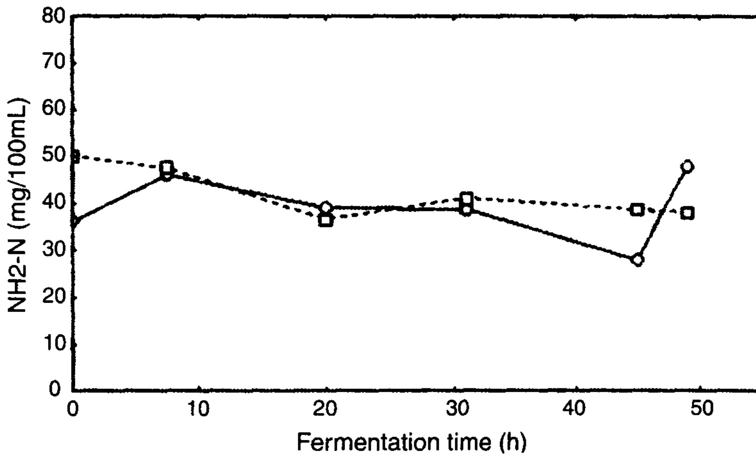

Fig. 6. Comparison of NH_2-N profiles between MDF (□) and BF (○).

and 2.85 U/mL · h respectively. If calculated on the basis of total enzyme activity, the enzyme activity of MDF was 33% higher than that in BF.

As shown in Figs. 5 and 6, the profiles of pH and NH_2-N in MDF were smoother than that in BF. The rise in pH and NH_2-N at the end of BF suggested that the culture had become degenerated.

Comparison of the Effect of Various Factors on the Enzyme Activity Between High- and Low-Yield Strains

Effect of Membrane Pore Size on GOD Synthesis in MDF

The results listed in Table 1 show that the larger the pore size of the membrane, the higher the enzyme activity, so the pore size of the mem-

Table 1
Effect of Membrane Pore Size on GOD synthesis

Membrane pore size (μm)	Enzyme activity of low yield strain (U/ mL)	Relative enzyme activity (%)[a]	Enzyme activity of high yield strain (U/mL)	Relative enzyme activity (%)[a]
Control[b]	23.07	100	54.60	100
0.3	31.25	135		
0.45	35.43	153		
0.65	42.04	182	68.94	126
0.80			75.64	139

[a] Relative enzyme activity = enzyme activity with MDF × 100 /control enz. activity.
[b] Control = batch fermentation.

Table 2
Effect of Initiating Time of Membrane Dialysis on Enzyme Production

Initiating time for dialysis (h)	Enzyme activity of low yield strain (U/ mL)	Relative enzyme activity (%)[a]	Enzyme activity of high yield strain (U/ mL)	Relative enzyme activity (%)[a]
Control[b]	23.08	100	54.1	100
20	24.68	107		
24	36.21	157	36.46	59
30	22.83	99	75.64	139
51			59.85	113

[a] Relative enzyme activity = enzyme activity with MDF × 100/control enz. activity,
[b] Control = batch fermentation.

brane should be as large as possible, provided that the cell can be retained within the fermenter. The positive effect of membrane dialysis using high-yield strain was less apparent than that using low-yield strain, which suggested that the high-yield strain could withstand a harsher environment than the low-yield strain.

Effect of Initiating Time for Membrane Dialysis on Enzyme Production

As shown in Table 2, if the membrane dialysis started too early or too late, the expected positive results could not be attained because of the loss of nutrients and useful metabolites in the former case, and the accumulation of detrimental metabolites in the latter case. Low-yield strain was more susceptible to the initiating time for membrane dialysis.

Effect of Volume of Dialysate on Enzyme Synthesis

The results displayed in Table 3 show that the total enzyme activity enhanced with the increase of dialysate volume. However, a large volume

Table 3
Effect of Dialysate Volume on Enzyme Synthesis

Dialysate volume (L)	Total enzyme activity in culture broth (U)	Total enzyme activity in dialysate (U)	Total enzyme activity (U)	Relative enzyme activity[a] (%)
Control[b]	42160	0	42160	100
1.5	38505	14280	52785	125
3.0	39645	26580	66225	157
6.0	39060	28320	67380	160

[a] Relative enzyme activity = enzyme activity with MDF × 100/Control enz. activity.
[b] Control = batch fermentation.

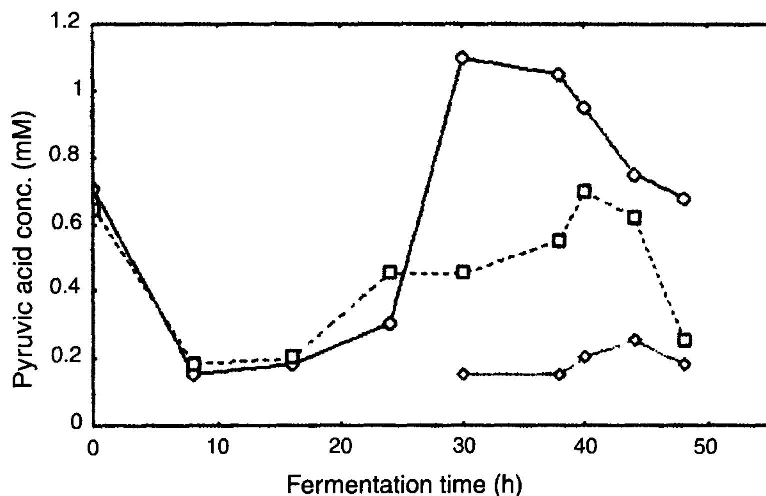

Fig. 7. Comparison of the profiles of pyruvic acid among BF (○), MDF (□), dialysate (◇).

of dialysate was unfavorable to downstream process, though the total enzyme activity did not decline. Therefore, the volume ratio of dialysate to culture broth = 2 : 1 was adopted throughout the experiment.

Application of Membrane Dialysis to the Study of the Effect of Metabolites on GOD Synthesis

Comparison of the Effect of Pyruvic Acid on the Production of GOD in BF and MDF

The results depicted in Fig. 7 show that before dialysis started the concentration of pyruvic acid reached 0.45 mmol at 24 h after inoculation. It remained at this level for 6 h after dialysis had started, and was raised to 0.55 mmol at 38 h of fermentation. In contrast, the concentration of pyruvic

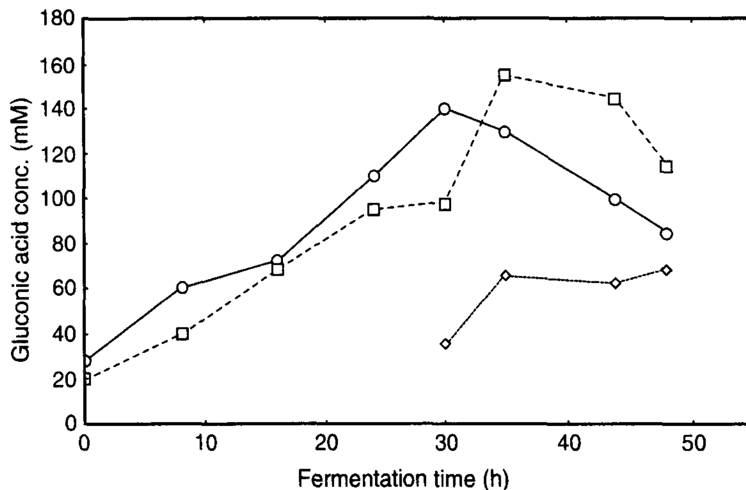

Fig. 8. Time-courses of gluconic acid concentration in BF (○), MDF (□), and dialysate (◇).

acid rose sharply from 0.30 mmol (at 24 h of age) to 1.10 mmol (at 30 h of age) and was maintained nearly at this level for 8 h in BF. As shown in Fig. 4, the peak of the production phase was between 30 and 38 h. Thus, it was disadvantageous to the enzyme production when the concentration of pyruvic acid was rather high at this period.

Effect of Gluconic Acid Concentration on Enzyme Production in BF and MDF

The time-courses of gluconic acid concentration in BF and MDF were shown in Fig. 8. In general, the influence of gluconic acid on GOD production was insignificant. At 30-h age of fermentation, the concentration of gluconic acid in MDF was lower than that in BF. It might have something to do with the increase in the enzyme production in MDF. However, gluconic acid concentration rose significantly at 35 h of fermentation. This might be because of the supplement of 2% (w/v) glucose in the dialysate. When the residual glucose in fermenter declined to lower than 2% (w/v), the glucose in dialysate diffused through the membrane to the fermenter, and this was the reason that the concentration of gluconic acid in MDF was higher than that of BF. At late phase of fermentation, the decline of gluconic acid might be the consequence of the consumption of it as a carbon energy source.

Analysis of Amino Acids in Culture Broth and Dialysate in MDF

The analysis of amino acids was performed with Hitachi 835-50 Model amino acid analyzer. Samples were withdrawn at 0, 16, 40, and 48 h in the fermenter and at 48 h in the dialysate reservoir. The results listed in Table 4 reveal that at 0 h of fermentation, 17 amino acids were detected in the sample. It seems that there exist some rules in the metabolism of amino acids during MDF. Whatever amino acid it was, it declined to its valley at

Table 4
Results of the Analysis of Amino Acids at Various Time of Fermentation in MDF

Amino acid	0-h fermentation broth		16-h fermentation broth		40-h fermentation broth		48-h fermentation broth		48-h reservoir dialysate	
	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)
Tau	0.384	100	0.354	92	0.099	26	0.141	37	0.092	24
Asp	0.894	100	0.779	87	0.140	16	0.462	52	0.186	21
Thr	0.601	100	1.154	192	0.237	39	3.286	547	0.289	48
Ser	0.824	100	0.723	88	0.127	15	0.852	104	0.101	12
Glu	0.901	100	2.056	228	0.519	58	1.829	203	0.649	72
Pro	0.991		-		-		-		-	
Gly	7.084	100	5.533	78	0.535	8	2.85	40	0.709	10
Ala	3.951	100	2.412	61	0.397	10	4.364	110	0.441	11
Cys	-		-		-		-		-	
Val	1.673	100	1.711	102	0.562	34	1.850	111	0.686	41
Met	0.712	100	0.502	71	0.480	67	1.031	145	0.393	55
Ile	0.335	100	0.528	158	0.175	52	0.533	159	0.210	63
Leu	1.295	100	1.746	135	0.301	23	1.354	105	0.378	29
Tyr	0.938	100	0.896	96	-		0.532	57	-	
Phe	2.616	100	2.666	102	1.667	64	2.280	87	1.655	63
Orn	0.331	100	-		0.297	90	-		0.262	88
Lys	0.647	100	0.584	87	0.214	33	0.449	69	0.274	42
His	0.542	100	0.407	75	0.090	17	0.550	102	0.119	22
Trp	-		-		-		-		-	
Arg	3.163	100	0.502	75	0.480	17	1.031	82	0.939	25

40 h of fermentation, and rose thereafter within 8 h to different extents. With the exception of Ile, the ascent of Thr, Glu, Ala, Val, Met, Leu, and His was more significant. Their contents were all greater than that at 0 h.

Sample was drawn at 48 h in a dialysate reservoir for the analysis of amino acid content. Among the amino acids analyzed, the contents of Phe and Arg showed the highest of all. According to article of Frederick *(13)*, the contents of these two amino acids in the constituents of GOD from *Aspergillus niger* are relatively low, so they are unlikely to be the limiting factors in GOD synthesis. Moreover, these two amino acids showed an adverse effect on the biosynthesis of GOD in the resting cell-culture system *(12)*. Thus, the decline of GOD productivity in late phase of batch fermentation might be attributed to the accumulation of Phe and Arg, and membrane dialysis could alleviate the accumulation of these two amino acids, so this might be the reason why MDF was superior to BF.

In addition, the contents of Ala and Gly in GOD molecule were relatively high *(13)*, and it was found that they all had a positive effect on GOD synthesis in the resting cell-culture system *(12)*. Fortunately, the contents of these two amino acids were relatively high in the initial medium and could be maintained at a high level in the fermenter until the end of MDF, but were rather low in dialysate, which meant that this was one of the reasons why MDF was better than BF.

Analysis of Organic Acids in Dialysate

The dialysate sample drawn at 48 h of fermentation was analyzed with Model Dionex 2010i Ionic Chromatography. The results (figure not shown) show that gluconic acid, which was predominant in the dialysate, contained 88 m*M*, and pyruvic acid was <0.6 m*M*, which was identical to the chemical assay method. Pyruvic acid and gluconic acid were unfavorable to GOD synthesis *(12)*. The results shown in Figs. 7 and 8 demonstrate it was advantageous to the enzyme synthesis that these two organic acids were permeated through the membrane to the dialysate during the production phase of GOD.

CONCLUSION

MDF can be applied to enhance the production of GOD. The results show that when MDF was employed, the enzyme volumetric productivity was two times higher than that of the control (fermentation without dialysis), and the total enzyme activity was increased by 30–50%. The appropriate operation conditions for MDF were as follows: pore size of the membrane, 0.65–0.80 µm; initiating time for membrane dialysis, 24–30 h; the volume ratio of dialysate to fermentation broth, 2.0–3.0. The profiles of pH and NH_2-N in MDF were smoother than that in BF. The rise in pH and NH_2-N at the end of fermentation suggested that the culture in BF had become degenerated. Seventeen amino acids were found in the medium. Phe, Arg, pyruvic acid, and gluconic acid exhibited an adverse effect, whereas Ala and Gly showed a positive effect on GOD production. The relationship among the content of pyruvic acid, gluconic acid, and enzyme activity during fermentation was quantitatively analyzed. The effect of membrane dialysis technology applied to the low-yield strain was proved to be more effective than that applied to the high-yield strain.

ACKNOWLEDGMENT

The authors gratefully acknowledge the support of this work (39170020) by the National Natural Science Foundation of China.

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