Biotechnol. Bioprocess Eng. 2001, 6: 11-17

Fed-batch Fermentation for Production of Nitrile Hydratase by *Rhodococcus rhodochrous* M33

Bu-Youn Kim[1], Jong-Chul Kim[1], Hyune-Hwan Lee[2], and Hyung-Hwan Hyun[2]*

[1] R&D Center, Tong Suh Petrochemical Corp. Ltd., P.O. Box 50, Nam-Ulsan, Korea
[2] Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Kyunggi-do 449-791, Korea

Abstract To enhance the productivity and activity of nitrile hydratase in *Rhodococcus rhodochrous* M33, a glucose-limited fed-batch culture was performed. In a fed-batch culture where the glucose was controlled at a limited level and cobalt was supplemented during the fermentation period, the cell mass and total activity of nitrile hydratase both increased 3.3-fold compared to that in the batch fermentation. The productivity of nitrile hydratase also increased 1.9-fold compared to that in the batch fermentation. The specific activity of nitrile hydratase in the whole cell preparation when using a fed-batch culture was 120 units/mg-DCW, which was similar to that in the batch culture.

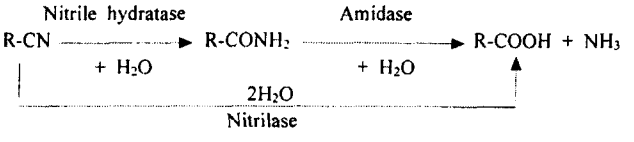
Keywords: nitrile hydratase, fed-batch culture, high-cell-density culture, *Rhodococcus rhodochrous* M33, acrylamide

INTRODUCTION

Acrylamide is a commodity chemical which is used as a starting material for the production of various polymers used as flocculants or stock additives, or for petroleum recovery or waste water treatment, and so forth. Acrylamide was originally produced by the chemical hydration of acrylonitrile with sulfuric acid in the presence of reduced copper as a catalyst [25]. However, chemical hydration reactions involve several problems, such as complexity in the preparation of the catalyst, difficulties in the purification and recovery of the acrylamide formed, the formation of by-products, a low conversion yield, and severe reaction conditions [8].

Recently, biotransformation processes to produce acrylamide from acrylonitrile using microorganisms with nitrile hydratase activity have been developed [11]. In the microbial hydration of nitrile, more than 99% of the acrylonitrile can be converted into acrylamide without the formation of by-products, plus the enzyme reaction can be carried out under moderate conditions. Several groups of bacteria such as *Rhodococcus, Pseudomonas, Bacillus, Corynebacterium, Brevibacterium* and *Arthrobacter* are known to be able to convert nitriles into corresponding amides [3,12,14,15,20,21].

Many microorganisms can use nitriles as a source of carbon and/or nitrogen for their growth, and the microbial nitrile metabolism is now well understood [5,6]. Aromatic, heterocyclic and certain unsaturated aliphatic nitriles are converted directly into corresponding acids and ammonia by nitrilase (EC 3.5.5.1, nitrile aminohydrolase) with little formation of free amide. In contrast, saturated aliphatic nitriles are catabolized in two stages; first, they are converted into corresponding amides by nitrile hydratase and then into acids and ammonia by amidase [7] as shown below.



Some nitrilase enzymes require an additional chemical component called a cofactor for their activity. Such cofactors can be either inorganic ions, such as Fe^{2+}, Mn^{2+} and Zn^{2+}, or a complex organic molecule. Nitrile hydratases also require a cofactor for their activity. It has been known that the nitrile hydratases from *Rhodococcus* sp. N774, *Pseudomonas chlororapis* B23 and *Brevibacterium* sp. R312 contain iron atoms as a cofactor [10,12,19]. These nitrile hydratases were the first non-heme iron enzymes discovered to contain typical low-spin ferric active sites [17,18].

However, not all nitrile hydratases contain iron. The nitrile hydratases from *Nocardia rhodochrous* LL100-21, *R. rhodochrous* J1, *R. rhodochrous* MO, and *R. rhodochrous* M33 contain cobalt atoms as a cofactor [1,13 23]. Cobalt atoms are tightly bound to the protein and no other transition metals can replace them. *Arthrobacter* sp. IPCB-3 has been found to produce about 2 fold and 4.5 fold more nitrile hydratase, respectively when iron and cobalt atoms are added to the medium [15].

Accordingly nitrile hydratases would appear to be either inducible or constitutive depending on the microorganism. Nitrile hydratases have been constitutively synthesized in *Brevibacterium* sp. R312, *Brevibacterium*

*Corresponding author
Tel: +82-31-330-4279 Fax: +82-31-333-1696
e-mail: hhhyun@san.hufs.ac.kr

sp. CH2, and *Rhodococcus* sp. N774, whereas the production of nitrile hydratases by *P. chlororaphis* B23 and *R. rhodochrous* J1 has been induced by inducers. *R. rhodochrous* J1 is known to produce one nitrilase and/or two nitrile hydratases depending on the inducer [8]. The occurrence of a complete change in the metabolic pathway based on the presence of a particular inducer in a single strain is a rare phenomenon.

Recently, fed-batch fermentations have been widely applied to obtain a high cell density in various microorganisms. The advantages of high-cell-density cultivation include reduced reactor volumes, less effort in upstream and downstream processing, higher volumetric productivity, easier preparation of cell suspension, improved yield in product recovery, reduced waste water, and lower production and investment costs [9,22]. Hwang and Chang [3] isolated *Brevibacterium* sp. CH1 to produce nitrile hydratase from the soil arround a acrylonitrile-producing plant, thereby developing a fed-batch fermentation processe. Choi *et al.* [2] also carried out the fed-batch culture of *Brevibacterium* sp. CH2 to obtain a high cell concentration.

This study performed a glucose-limited fed-batch culture of *Rhodococcus rhodochrous* M33 to improve the productivity and total activity of its nitrile hydratase.

MATERIALS AND METHODS

Microorganism

The *Rhodococcus rhodochrous* M33 was obtained from the Institute for Genetics for Microorganisms, VNII Genetika (Russia).

Media and Culture Conditions

For the preparation of a frozen stock, a culture broth of *R. rhodochrous* M33 was mixed with 30% glycerol at a ratio of 1:1, frozen, and then, stored in a deep freezer (-80°C). To prepare the seed culture, cells taken from the frozen stock were transferred to a 500 mL Erlenmeyer flask containing 50 mL of a seed medium consisting of 0.5 g of KH_2PO_4, 0.4 g of K_2HPO_4, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of EDTA-2Na, 20 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 3 g of urea per liter of demineralized water, and cultivated at 30°C for 48 h with shaking at 500 rpm. Thereafter, the seed culture was transferred to a 5-L jar fermenter (Korea Fermenter Co., Incheon, Korea) containing 3 L of the same medium as used in the seed cultures, with the exception that 0.01 g/L of NaCl, 7.5 g/L of urea, 1.2 g/L of $MgSO_4 \cdot 7H_2O$, and 0.018 g/L $CoCl_2 \cdot 6H_2O$ were supplemented to the medium. The Batch fermentations were carried out for 50 h in the same jar fermentor which was controlled at 30°C, agitated at 500 rpm, and aerated at 0.4 vvm.

The fed-batch fermentations were carried out in a 5-L jar fermenter equipped with a dissolved oxygen (DO) analyzer (Mettler Toledo AG, Switzerland) and pH controller. A feed solution consisting of 40% glucose and other components such as urea and $CoCl_2 \cdot 6H_2O$, was fed to the fermentor either intermittently whenever the glucose in the fermentation broth was almost exhausted or continuously using a peristaltic pump (MasterFlex, USA) so that the glucose concentration in the culture broth was maintained at a limited level. The dissolved oxygen concentration(DO) was maintained above 10% of air saturation by controlling the agitation speed and air flow rate within a range of 500 and 700 rpm, and 0.4 and 1.2 vvm, respectively, after the optical densities of the culture broth at 540 nm reached 40 to 50. The pH in the fermentor was controlled at 7.2 using a 10% KOH solution and the culture temperature was maintained at 30°C. An antifoam (polyoxyethylene-oxypropylene glycol ether) was added when necessary. The cells were then harvested by centrifugation at 8,000 rpm at 4°C and washed twice with demineralized water.

Assay of Nitrile Hydratase Activity

To measure the nitrile hydratase activity in the cell suspensions, a reaction mixture (2 mL) consisting of a 1% (w/w) acrylonitrile solution in a 12.5 mM phosphate buffer (pH 7.5) and 0.04 mg of cells (dry cell weight) was incubated at 20°C for 10 min with moderate shak-ing, then the reaction was stopped by adding 40 μL of conc. HCl. The amount of acrylamide produced in the reaction mixture was determined using a gas chromatography (AutoSystem GC, Perkin Elmer, USA).

One unit of nitrile hydratase activity was defined as the amount of the whole cell enzyme that catalyzed the formation of 1 μmol of acrylamide per min under the above reaction conditions. The specific activity of the nitrile hydratase was expressed as units per mg of dry cells.

Analytical Methods

To determine the culture turbidities, the culture broths were appropriately diluted with demineralized water, then the optical densities were measured at 540 nm using a UV-Visible spectrophotometer (Cary, Varian Co., Australia). The dry cell weight (DCW) was estimated using a calibration curve based on the relationship between the optical density at 540 nm and the dry cell weight, and 1.0 OD_{540} was considered as equivalent to 0.2 g dry cell weight/L. The glucose concentration was enzymatically determined using a glucose biochemistry analyzer (YSI model 2700, Yellow Spring Instrument Co., Inc., USA).

The amount of nitrile consumed and amide formed in the reaction mixture were determined using a gas chromatography (AutoSystem GC, Perkin Elmer, USA) equipped with a frame ionized detector. The column used was a stainless steel column packed with a 8% free fatty acid phase for the amide analysis and Porapak Type Q (80-100 mesh) for the nitrile analysis. The detector and injector temperature was 230°C and the col-

umn temperature was 190°C. The carrier gas was N_2 and its flow rate was 80 mL/min. The integration and calibration of the peak areas were carried out with a PE-NELSON model 1020.

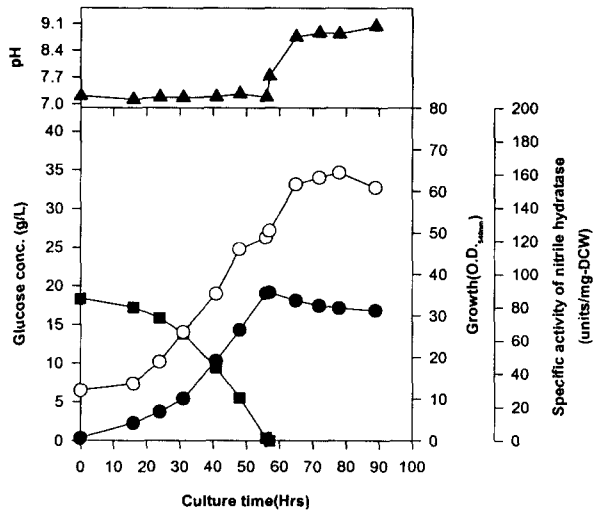
Chemicals

The medium components were usual commercial products and used without further purification. All other chemicals were of reagent grade and purchased from Aldrich Chemical Company, Inc., USA.

RESULTS AND DISCUSSION

Nitrile Hydratase Production by Batch Fermentation

The batch culture of *R. rhodochrous* M33 used to obtain the fundamental fermentation parameters in relation to the nitrile hydratase production was performed in a 5-L jar fermentor. The glucose fermentation time course is shown in Fig. 1. The maximum cell concentration, cell yield coefficient on glucose ($Y_{x/s}$), and maximum specific growth rate was 7.1 g-DCW/L, 0.36 g-cell/g-glucose and 0.08 h^{-1}, respectively. The nitrile hydratase production was found to be closely associated with growth. The specific activity of nitrile hydratase in cells increased in proportion to growth until 24 h after the glucose was exhausted. After 78 h of cultivation, the specific activity of nitrile hydratase and the total nitrile hydratase activity was 160 units/mg-DCW and 1,140 units/mL, respectively. However, it was observed that the bacterial cells died gradually during the stationary growth phase when the glucose was completely consumed, plus the dead cells exerted a harmful influence on the biotransformation reaction of acrylonirile into acrylamide (data not shown). Therefore, it appeared desirable to harvest the cells immediately after the glucose was exhausted.



Fig, 1. Profiles of cell growth, nitrile hydratase production, pH, and glucose in batch fermentation of *R. rhodochrous* M33. -●-, growth; -■-, glucose; -O-, specific activity of nitrile hydratase; -▲-, pH.

Nitrile Hydratase Production by Fed-batch Fermentation

To enhance the total activity of nitrile hydratase, the specific activity of nitrile hydratase, and the cell density, fed-batch cultures of *R. rhodochrous* M33 were performed and the effect of the key fermentation parameters on the nitrile hydratase production by a fed-batch culture was monitored.

Effect of Dissolved Oxygen (DO) Concentration

DO is one of the important parameters in the cultivation of obligate aerobic bacteria. The effect of the DO concentration on the nitrile hydratase production in a fed-batch culture where the medium composition was the same as described in Materials and Methods and the feed solution was a 40% glucose solution was investigated. The glucose solution was intermittently fed into the fermentor whenever the glucose concentration in the culture medium went below 10 g/L, and the DO

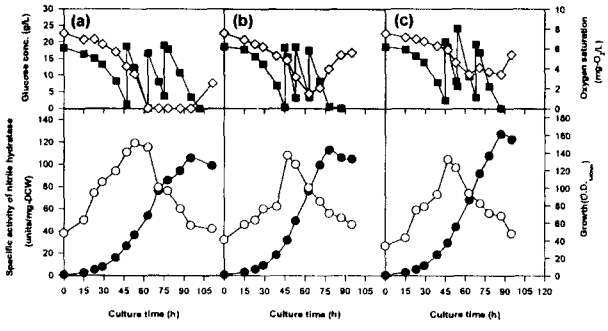


Fig. 2. Fed batch fermentations of *R. rhodochrous* M33 in which DO levels were differently controlled according to agitation speed and air flow rate. (a), The aeration rate and agitation speed were kept constant at 0.4 vvm and 500 rpm, respectively; (b), The aeration rate and agitation speed were increased from 0.4 to 0.8 vvm and from 500 to 700 rpm, respectively, at the time of the first glucose feeding; (c), The aeration rate and agitation speed were increased from 0.4 to 1.2 vvm and from 500 to 700 rpm, respectively, at the time of the first glucose feeding. -●-, growth; -■-, glucose; -O-, specific activity of nitrile hydratase; -◇-, DO.

level in the fermentor was controlled by regulating the agitation speed and air flow rate when the optical densities of the culture broth at 540 nm reached 40 to 50.

As shown in Fig. 2, the growth rate was not affected by the DO level, however, the highest cell concentration was obtained when the agitation speed and aeration rate were increased from 500 to 700 rpm and from 0.4 to 1.2 vvm, respectively (Fig. 2(c)). The specific activity of nitrile hydratase increased in proportion to the cell growth and the highest nitrile hydratase activity (120 units/mg-DCW) was obtained after 45 h when the

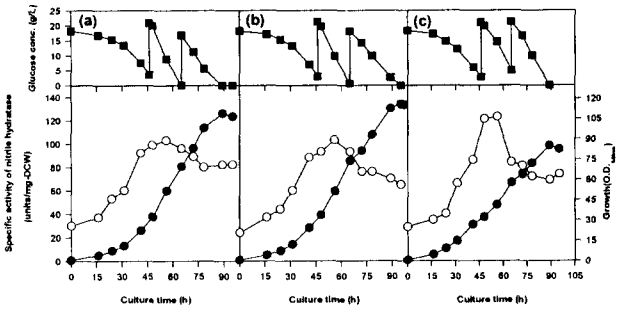


Fig. 3. Fed-batch fermentations of *R. rhodochrous* M33 in which different compositions of feed solutions were intermittently fed into the fermenter. (a), A feed solution containing 60 g of glucose was intermittently fed twice into the fermenter containing 3 liters of medium; (b), A feed solution consisting of 60 g of glucose and 9 g of urea was intermittently fed twice into the fermenter containing 3 liters of medium; (c), A feed solution consisting of 60 g of glucose and 0.03 g of $CoCl_2 \cdot 6H_2O$ was intermittently fed twice into the fermenter containing 3 liters of medium. -●-, growth; -■-, glucose; -O-, specific activity of nitrile hydratase.

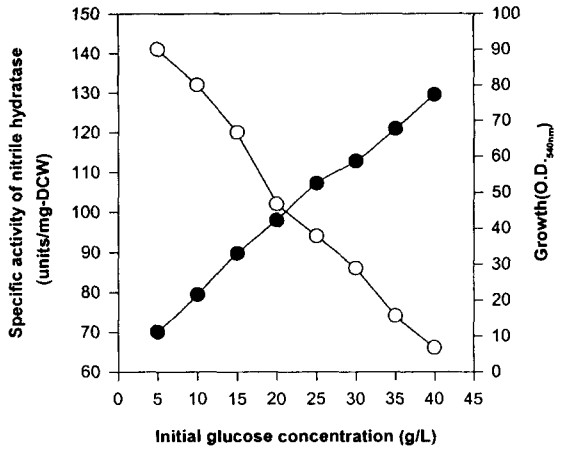


Fig. 4. Effect of initial glucose concentrations on the cell growth and the nitrile hydratase activity of *R. rhodochrous* M33. -●-, growth; -O-, specific activity of nitrile hydratase. The cells were grown at 30°C in a 500 mL-Erlenmeyer flask containing 50 mL of medium with shaking. The cell growth and enzyme activity were measured when the glucose in the culture broth was exhausted.

OD_{540} of the culture broth reached about 50. Yet after 45 h of culture time, the specific activity of nitrile hydratase gradually decreased to a final level of about 40 units/mg-DCW. Accordingly, it was concluded that the specific enzyme activity was unaffected by the DO level, however, an increase in the agitation speed and air flow rate from 500 to 700 rpm and from 0.4 to 1.2 vvm, respectively, was found to be desirable to obtain the highest cell density.

Effect of Feed Composition

The nitrile hydratase of *R. rhodochrous* M33 is known to contain cobalt atoms as a cofactor, plus its production is stimulated by urea [24]. Therefore, it was considered that the gradual decrease of the specific activity of nitrile hydratase in the fed-batch cultures may have been due to a lack of either cobalt ions or urea in the culture broth. In order to determine whether or not the nitrile hydratase production could be enhanced by the addition of cobalt or urea in the fed-batch culture, a glucose solution containing urea or $CoCl_2 \cdot 6H_2O$ was used as the feed solution. Fig. 3 indicates that the cell growth rate was not affected by the addition of cobalt, however, the highest specific activity of nitrile hydratase (120 units/mg-DCW) was obtained when cobalt was added to the feed solution although the final cell concentration was lower than those cases when no cobalt was added. However, the addition of urea did not severely affect the growth rate, the specific activity of nitrile hydratase and the final cell concentration. These results would seem to indicate that cobalt ions have a positive effect on the synthesis of the enzyme yet a negative effect on the cell growth. However, the addition of cobalt was unable to overcome the problem that the specific activity of nitrile hydratase gradually decreased after 55 h of culture time.

Effect of Glucose

To improve the productivity of the fermentation products by derepression, metabolic shift and so on, a cell culture is frequently carried out under unfavorable growth conditions, such as C-source limitation, N-source limitation, K-limitation, Mg-limitation, P-limitation, and temperature shifts [4,16].

Fig. 4 shows the effect of the initial glucose concentration within a range of 5 g/L to 40 g/L on the cell growth and specific activity of nitrile hydratase in *R. rhodochrous* M33. It was found that the final cell concentration increased in proportion to the initial glucose concentration, whereas the specific activity of nitrile hydratase decreased inversely. These results indicate that the production of nitrile hydratase may be subject to catabolite repression by glucose.

Based on these results, fed-batch cultures were carried out, in which the glucose concentration in the culture broth was maintained at an almost constant level (limited level, 5 g/L and 10 g/L) during the fermentation by continuously feeding a glucose solution in proportion to its consumption. As shown in Fig. 5, the lower the glucose concentration in the culture broth, the slower the growth and the higher the specific activity of nitrile hydratase. Therefore, it was concluded that the limitation of glucose in the culture broth resulted in an enhanced production of nitrile hydratase.

Effect of Cofactor

The effect of the initial cobalt concentration on the cell growth and enzyme activity was investigated, as shown in Table 1. Within an initial $CoCl_2 \cdot 6H_2O$ concentration range of 0.005-0.030 g/L, cell growth was almost similar, however, the specific activity of nitrile hydratase increased in proportion to an increase in the

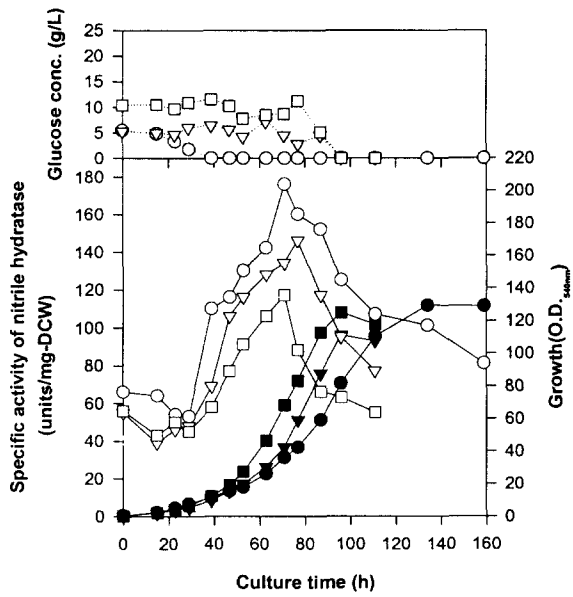


Fig. 5. Comparison of fermentation time courses in fed-batch cultures of *R. rhodochrous* M33 in which the glucose concentrations in the culture broths were maintained at a limited level, 5 g/L and 10 g/L, respectively, by regulating the glucose feeding rate. circle, limited level (-●-, growth; ···○···, glucose; -○-, specific activity of nitrile hydratase); triangle, 5 g/L (-▼-, growth; ···▽···, glucose; -▽-, specific activity of nitrile hydratase); square, 10 g/L (-■-, growth; ···□···, glucose; -□-, specific activity of nitrile hydratase).

Table 1. Effect of initial cobalt concentrations on cell growth and nitrile hydratase production in *R. rhodochrous* M33a

Initial $CoCl_2 \cdot 6H_2O$ conc. (g/L)	Growth (OD_{540nm})	Specific activity (units/mg-DCW)
Not added	29.5	12
0.005	41.6	30
0.010	38.5	32
0.015	33.2	60
0.020	32.6	65
0.025	37.5	86
0.030	34.3	121
0.035	26.6	104
0.040	11.6	90

[a] Cells were grown in a 500 mL-Erlenmeyer flask containing 50 mL of the main culture medium at 30°C for 49 h with shaking.

initial $CoCl_2 \cdot 6H_2O$ concentration. The maximum specific activity of nitrile hydratase was recorded at 121 units/mg-DCW with a 0.030 g/L initial $CoCl_2 \cdot 6H_2O$ concentration, then above this concentration, the specific activity of nitrile hydratase and cell growth both decreased. When cobalt was not added, the specific activity of nitrile hydratase was very low although growth was excellent. These data coincide with previous reports that noted that cobalt atoms play the role of a cofactor in the nitrile hydratase of *R. rhodochrous* M33 [23,24].

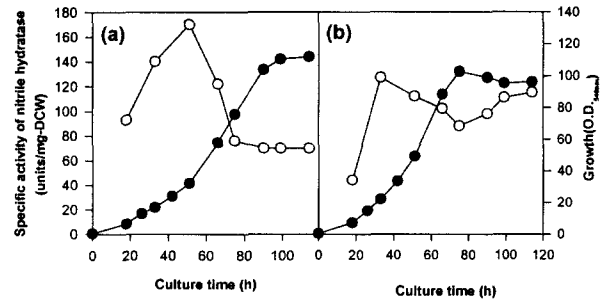


Fig. 6. Comparison of fermentation time courses in glucose-limited fed-batch cultures of *R. rhodochrous* M33 in which 0.01 g/L of $CoCl_2 \cdot 6H_2O$ was supplemented (b) or not (a) into the glucose feed. -●-, growth; -○-, specific activity of nitrile hydratase.

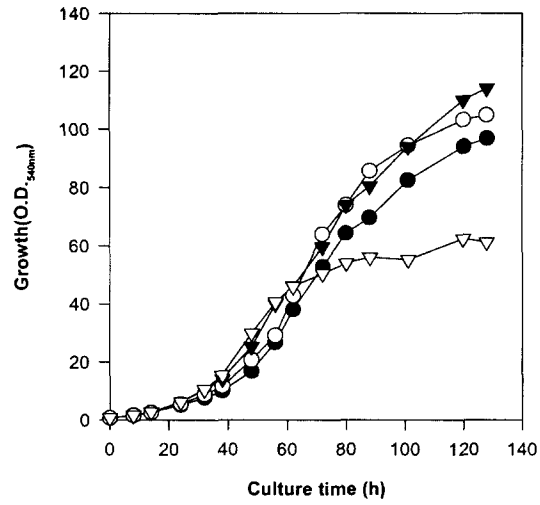


Fig. 7. Effect of cobalt concentration in feed solution on cell growth in glucose-limited fed-batch culture of *R. rhodochrous* M33. -●-, Not added; -○-, $CoCl_2 \cdot 6H_2O$ 0.005 g/L ; -▼-, $CoCl_2 \cdot 6H_2O$ 0.01 g/L; -▽-, $CoCl_2 \cdot 6H_2O$ 0.02 g/L.

In order to overcome the problem that the specific activity of nitrile hydratase gradually decreased during the late fermentation period, the effect of feeding cobalt on the synthesis, activity, or stability of the enzyme was investigated using a glucose-limited fed batch culture. Fig. 6 illustrates that the final specific activity of nitrile hydratase was enhanced 1.5-fold by the addition of cobalt (0.01 g/L) to the feed solution.

Accordingly, glucose-limited fed-batch cultures which were continuously fed with a glucose solution containing various amount of $CoCl_2 \cdot 6H_2O$ were conducted to identify the optimal concentration level of cobalt for the feed solution. As shown in Fig. 7, cell growth was severely inhibited when a feed solution containing 0.02 g/L of $CoCl_2 \cdot 6H_2O$ was continuously fed into the fermentation medium. Therefore, it was concluded that a glucose-limited fed-batch culture in which a glucose solution is continuously fed into the fermentor and $CoCl_2 \cdot 6H_2O$ is intermittently fed into the glucose feed

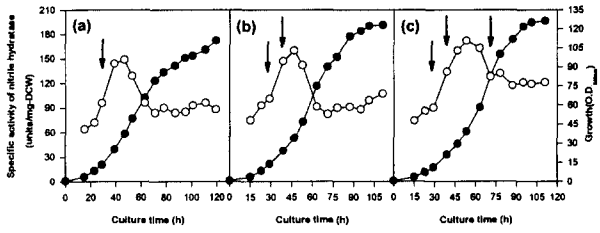


Fig. 8. Effect of cobalt addition to feed solution on cell growth and specific activity of nitrile hydratase in glucose-limited fed-batch cultures of *R. rhodochrous* M33. Arrows indicate the addition of $CoCl_2 \cdot 6H_2O$ (0.01 g/L) to the feed solution. -●-, growth; -O-, specific activity of nitrile hydratase.

solution at 0.01 g/L would appear to be desirable. The results shown in Fig. 8 indicate that both the maximum specific activity and the final specific activity of nitrile hydratase were increased by the intermittent addition of $CoCl_2 \cdot 6H_2O$ into the glucose feed. When 0.01 g/L of $CoCl_2 \cdot 6H_2O$ was fed three times into the glucose feed, the final specific activity of nitrile hydratase was estimated to be 120 units/mg-DCW which was almost equivalent to that of the batch culture.

Comparison of Batch and Fed-batch Fermentations

The kinetic parameters of the batch and fed-batch fermentations for the production of nitrile hydratase by *R. rhodochrous* M33 are compared in Table 2. It was found that the cell density, total activity of nitrile hydratase, and productivity were much higher in the fed-batch cultures than in the batch culture, yet the specific activity of nitrile hydratase was rather lower in the fed-batch cultures. In order to improve the specific activity of nitrile hydratase, the composition of the feed solution and the feeding method in the fed-batch cultures were modified. Consequently, the specific activity of nitrile hydratase was improved to the level obtained in the batch culture by the use of fed-batch cultures in which a glucose feed was continuously fed into the fermentor to maintain a limited level of glucose concentration in the culture broth and cobalt was intermittently fed into the glucose feed. The reason for such an increase is still unclear, however, it is presumed that the enhanced synthesis of nitrile hydratase resulted from the removal of catabolite repression and the supplementation of a cobalt deficiency.

The cell mass and total activity of nitrile hydratase in a glucose-limited fed-batch culture in which cobalt was added three times to the glucose feed was 24 g-DCW/L and 2,880 units/mL, respectively, and both were 3.3-fold higher in comparison with those in the batch culture. The productivity of nitrile hydratase in the fed-batch cultures was also 1.9-fold higher at 28.5 units mL^{-1} h^{-1} compared to those in the batch culture. The specific activity of nitrile hydratase was 120 units/mg-DCW, which was almost equivalent to that in the batch culture.

Acknowledgements The authors are grateful to K. C. Lee (President of Tong Suh Petrochemical Corp. Ltd.), J. P. Huh (Manager of Plant) and G. S. Park (Manager of R&D) for their support and permission for the publication of this work. The competent technical assistance by Mr. H. L. Kim is also acknowledged.

REFERENCES

[1] Astaurova, O. B., T. E. Pogorelova, O. R. Fomina, I. N. Polyakova, and A. S. Yanenko (1991) Regulation of the biosynthesis of nitrile biodegradation enzymes in *R.hodococcus rhodochrous* MO. *Biotekhnologiya.* 5: 10-14.

[2] Choi, S. K., C. Y. Lee, H. N. Chang, and J. S. Hwang (1991) Optimum culture conditions of *Brevibacterium* sp. CH2 for production of nitrile hydratase. *J. Microbiol. Biotechnol.* 1: 136-141.

[3] Hwang, J. S., and H. N. Chang (1992) Fed-batch culture of *Brevibacterium* CH1 for the production of nitrile

Table 2. Comparison of kinetic parameters of batch and fed-batch cultures for production of nitrile hydratase by *R. rhodochrous* M33

Mode of operation	Cultivation conditions	Cell mass (g-DCW/L)	Cell yield($Y_{X/S}$) (g-cell/g-glucose)	Specific activity (units/mg-DCW)	Total activity (units/mL)	Productivity (units mL^{-1} h^{-1})
Batch	Glucose 20 g/L	7.1	0.35	120	852	14.7
Fed-batch	Intermittent feeding of glucose (80 g/L)	32.0	0.40	50	1600	17.4
	Constant feeding of glucose (60 g/L)	24.0	0.40	80	1920	19.2
	Constant feeding of glucose (60 g/L) & cobalt additions	24.0	0.40	120	2880	28.5

hydratase. *Kor. J. Appl. Microbiol. Biotech.* 20: 614-618.
[4] Kim, H. G., T. H. Kim, D. S. Kim, K. Y. Park, J. S. Park, C. Ahn, J. H. Lee, and H. Y. Lee (1996) The effect of glutamine on production of tissue-type plasminogen activator from recombinant human melanoma cells in glutamine-limited fed-batch cultivation. *J. Microbiol. Biotechnol.* 6: 295-298.
[5] Knowles, C. J. (1976) Microorganisms and cyanide. *Bacteriol. Rev.* 40: 652-860.
[6] Knowles, C. J. (1985) Microbial degradation of cyanide. *Biotech '85 (Europ)*, 537-542.
[7] Knowles, C. J. and A. W. Bunch (1986) Microbial cyanide metabolism. *Adv. Microb. Physiol.* 27: 73-111.
[8] Kobayashi, M., T. Nagasawa, and H. Yamada (1992) Enzymatic synthesis of acrylamide: a success story not yet over. *Trends Biotechnol.* 10: 402-408.
[9] Lee, S. Y. (1996) High cell-density culture of *Escherichia coli*. *Tibtech March.* 14: 98-105.
[10] Nagasawa, T., H. Nanba, K. Ryuno, K. Takeuchi, and H. Yamada (1987) Nitrile hydratase of *Pseudomonas chlororaphis* B23: Purification and characterization. *Eur. J. Biochem.* 162: 691-698.
[11] Nagasawa, T. and H. Yamada (1989). Microbial transformations of nitriles. *Trends Biotechnol.* 7: 153-158
[12] Nagasawa, T., K. Ryuno, and H. Yamada (1986) Nitrile hydratase of *Brevibacterium* R312: Purification and characterization. *Biochem. Biophy. Res. Comm.* 139: 1305-1312.
[13] Nagasawa, T., K. Takeuchi, and H. Yamada (1991) Characterization of a new cobalt-containing nitrile hydratase purufied from urea-induced cells of *R. rhodochrous* J1. *Eur. J. Biochem.* 196: 581-589.
[14] Nagasawa, T., K. Takeuchi, V. Nardi-Dei, Y. Mihara, and H. Yamada (1991) Optimum culture conditions for the production of Co-containing nitrile hydratase *R. rhodochrous* J1. *Appl. Microbiol. Biotechnol.* 34: 783-788.
[15] Ramakrishna, C. and J. D. Desai (1992) Induction of iron and cobalt dependent nitrile hydratase in *Arthrobacter sp.* IPCB-3. *Biotechnol. Letter.* 14: 827-830.
[16] Ryu, H. W., S. K. Hahn, Y. K. Chang, and H. N. Chang (1997) Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of *Alcaligenes eutrophus* with phosphate limitation. *Biotechnol. Bioeng.* 55: 28-32.
[17] Sugiura, Y., and J. Kuwahara (1987) Nitrile hydratase: The first non-heme iron enzyme with a typical low-spin Fe(III)-active center. *J. Am. Chem. Soc.* 109: 5848-5850.
[18] Sugiura, Y., J. Kuwahara, T. Nagasawa, and H. Yamada (1988) Significant interaction between low-spin iron(III) site and pyrroloquinoline quinone in active center of nitrile hydratase. *Biochem. Biophy. Res. Comm.* 154: 522-528.
[19] Thiery A., M. Maestracci, A. Arnaud, P. Galzy, and M. Nicolas (1986) Pruification and properties of an acrylamide amidohydrolase(E. C. 3.5.1.4) with a wide activity spectrum from *Brevibacterium* sp. R312. *J. Basic Microbiol.* 5: 299-311.
[20] Watanabe, I., Y. Satoh, K. Enomoto, S. Seki, and K. Sakashita (1987) Optimal conditions for cultivation of *Rhodococcus* sp. N-774 and for conversion of acrylonitrile to acrylamide by resting cells. *Agric. Biol. Chem.* 51: 3201-3206.
[21] Yamada, H., K. Ryuno, T. Nagasawa, and I. Watanabe (1986) Optimum culture conditions for production of *P. chororaphis* B23 of nitrile hydratase. *Agric. Biol. Chem.* 50: 2859-2865.
[22] Yamane, T., M. Fukunaga, and L. Y. Woo (1996) Increased PHB productivity by high-cell-density fed-batch culture of *Alcaligenes latus,* a growth-associated PHB producer. *Biotechnol. Bioeng.* 50: 197-202.
[23] Yanenko A. S. (1998) Strain of *Rhodococcus rhodochrous* as a producer of nitrile hydratase. *US patent* 5,827,699.
[24] Yanenko A. S., O. B. Astaurova, T. V. Gerasimova, I. N. Polyakova, T. E. Pogorelova, and V. N. Paukov (1995) Regulation of nitrile utilization in *Rhodococcus. Proceedings of the Ninth Symposium on the Actinomycetes.* 139-144.
[25] Yen, C. Y. (1976) Acrylamide. SRI Report, Standford Research Institute, California. 99: 1-7.

[Received September 18, 2000; accepted February 16, 2001]