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Effects of Acrylonitrile and Acrylamide on Nitrile Hydratase Action of *Brevibacterium* sp. CH1 and CH2

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The effects of acrylonitrile and acrylamide on the enzyme action of nitrile hydratase of *Brevibacterium* sp. CH1 and CH2 strains used for the biotransformations of nitriles were studied. The excessive substrate (acrylonitrile) and product (acrylamide) inhibited the enzyme activity competitively. In comparison with 0.2 mol/l of CH1 strain, the substrate inhibition of CH2 strain began to appear only at a high acrylonitrile concentration of 0.91 mol/l. In a packed bed reactor, dispersed plug flow model was proposed and this model was proved to be valid by the experiment. Also acrylamide productivity decreased sharply when acrylamide concentration in the substrate solution exceeded 20% (wt/v).

Acrylamide is one of the most important commodity chemicals. Acrylamide is industrially used as monomer and additives for flocculant agents in sewage treatment, petroleum recovery, paper making, textile sizes and other industrial materials and its current world demand is about 200,000 tons per year. In conventional systhesis, the hydration of acrylonitrile uses copper salts as a catalyst and high acrylonitrile concentration of 7% (wt/v) as reactant. However, the catalyst preparation, the regeneration of the used catalyst and the separation and purification of acrylamide formed are laborious and the process requires a high temperature. Furthermore, because acrylamides are rapidly polymerized, their production under moderate conditions is desirable.

Several groups of bacteria such as *Nocardia, Brevibacterium, Arthrobacter, Rhodococcus, Corynebacterium,* and *Pseudomonas* are able to convert nitriles to corresponding amides (1, 2, 9-11, 16-18, 21).

The *Brevibacterium* sp. possess two enzymes involved in the metabolism of nitriles; —a nitrile hydratase hydrates water soluble nitriles into the corresponding amides, —an amidase hydrolyses the amides into corresponding acids and ammonia. The reaction is as follows (3, 4, 19, 20).

$$\text{R-CN} \xrightarrow[+H_2O]{\text{nitrile hydratase}} \text{R-CONH}_2 \xrightarrow[+H_2O]{\text{amidase}} \text{R-COOH} + NH_3$$

The specific activity of amidase is lower than that of nitrile hydratase. Therefore, nearly 100% of acrylonitrile is converted to acrylamide with only a trace amount of acrylic acid produced. This is a promising method for acrylamide production in comparison with the chemical hydration by copper-based catalyst in terms of selectivity, reaction temperature, pressure, and ease of the catalyst preparation.

However, the biological acrylamide production has following disadvantages; the substrate inhibition occurred only at a relatively low acrylonitrile concentration of 0.4 mol/l or less (5, 17). In comparison with chemical hydrolysis using the high concentration of acrylonitrile (7%), enzymatic transformation of acrylonitrile to acrylamide can not use the high concentration of acrylonitrile because of the substrate inhibition. The study of specific rates of bioconversion reactions of nitriles and amides (6), the inhibition of the nitrile hydratase enzyme and the action of some nitriles (7), and the excess of substrates and products on the activity of the amidase were previously reported (15).

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Key words: Acrylonitrile, acrylamide, substrate inhibition, packed bed reactor

Recently, we developed *Brevibacterium* sp. CH2 by repeated cultivation of *Brevibcterium* sp. CH1 in the broth with gradually increased acrylonitrile concentration. The specific nitrile hydratase activity of CH2 strain was 3.2 times higher than that of CH1 strain and the CH2 enzyme has a higher acrylonitrile concentration tolerance than that of the CH1 enzyme (14). In the present work, we describe the acrylonitrile and acrylamide inhibition of *Brevibacterium* sp. CH1 and CH2 strain and the analysis of packed bed reactor.

ENZYME REACTION KINETICS AND ANALYSIS OF PACKED BED REACTOR

Kinetics

If the nitrile hydratase of *Brevibacterium* sp. is inhibited competitively by the excess substrate and product, the reaction rate for the substrate and product inhibition can be modelled by the reaction mechanism of the Michaelis-Menten type.

Reaction steps at equilibrium:

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad K_m=\frac{k_1}{k_{-1}}$$

$$ES+S \rightleftharpoons ES_2 \qquad K_s \tag{1}$$
$$E+P \rightleftharpoons EP \qquad K_p$$

where, we assume that ES_2 and EP do not have reactivity.

Rate limiting step:

$$ES \xrightarrow{k} E+P \tag{2}$$

$$\text{Reaction rate, } V=k[ES] \tag{3}$$

If all four equilibria in eq. (1) are written algebraically, and all variable except total enzyme concentration ($[E_o]$), free substrate and product concentrations ([S] and [P] respectively) are removed, the results are follows:

$$V=\frac{V_{max}}{1+\frac{K_m}{[S]}+\frac{[S]}{K_s}+\frac{K_m}{K_p}\frac{[P]}{[S]}} \tag{4}$$

where, the maximum reaction rate $V_{max}=k[E_o]$
The product concentration [P] is given by

$$[P]=[P_o]+[S_o]-[S] \tag{5}$$

Dispersed Plug Flow Model

If the model system is stayed at steady state and packed bed reactor is operated as a plug flow reactor, the substrate balance in a packed bed reactor is given as follows:

$$u_s\frac{d[S]}{dz}=-(1-\varepsilon)V\eta W \tag{6}$$

$$\text{subject to} \quad z=0,\ [S]=[S_o] \tag{7}$$

$$z=L,\ [S]=[S_f] \tag{8}$$

Integration of eqs. (4), (5), and (6) with the boundary conditions established by eqs. (7) and (8) yield the substrate and product concentrations in the reactor with space time.

$$\left(1-\frac{K_m}{K_p}\right)([S_o]-[S_f])+K_m\left\{1+\frac{([S_o]+[P_o])}{K_p}\right\}\ln\frac{S_o}{S_f}+\frac{1}{2K_s}([S_o]^2-[S_f]^2)=\frac{1-\varepsilon}{\varepsilon}\tau\eta WV_{max} \tag{9}$$

where, $\tau=\frac{\varepsilon L}{u_s}$; $u_s=\frac{Q}{A}$;

ε is void fraction; η refers to effectiveness factor; u_s is superficial velocity; W is the amount of the immobilized cells (dry weight); $[S_o]$ is the substrate concentration in the feed; L is the reactor length; $[S_f]$ is the substrate concentration in the effluent; $[P_o]$ is the product concentration in the feed; Q is feed rate; A is cross-sectional area of the reactor; and τ is space time.

MATERIALS AND METHODS

Microorganism

The strains used were *Brevibacterium* sp. CH1 and CH2 strain. CH1 strain was isolated from soil (8) and CH2 strain was developed by acrylonitrile adaptation from CH1 strain (14).

Medium and Culture Conditions

The basic culture medium is a synthetic medium containing the following components, per liter of distilled water: glucose 15 g; yeast extract 3 g; malt extract 3 g; bacto peptone 5 g; K_2HPO_4 1 g; KH_2PO_4 1 g; NaCl 1 g; $MgSO_4 \cdot 7H_2O$ 0.2 g. It was sterilized in an autoclave at 121℃ for 15 min.

The seed culture was inoculated with *Brevibacterium* sp. in a 250 ml Erlenmeyer flask containing 50 ml of medium and cultivated in a rotary shaker with 300 rpm at 30℃ for 24 h. The main culture was carried out in a 1.5 *l* fermenter (Biostat E, B. Braun Co.) at 30℃, pH 7 and 500 rpm. Aeration was performed by filtered air into the vessel at 2 vvm.

Reaction Conditions

In this paper, standard reaction conditions were defined as pH 7.0 and 4℃. Acrylonitrile solution was prepared by adding in a 0.1 M Na_2HPO_4-NaH_2PO_4 buffer. Reaction was carried out under these reaction conditions with moderate shaking after the cells were suspended in a 0.1 M phosphate buffer. Reaction velocity was assayed by measuring the specific activity of nitrile hydratase. Specific activity was measured using a method similar to that previously described (14). One unit of nitrile hydratase activity was defined as the amount of the whole

cells that catalyzed the formation of 1 μmols of acrylamide per min at the standard reaction conditions. The specific activity and reaction velocity were expressed as units/mg of dry cells and (units/mg of dry cells)$^{-1}$, respectively.

Assays

Acrylamide and acrylonitrile were measured by gas chromatography with a chromosorb W (80~100 mesh) coated with carbowax 20 M 10% and flame ionization detector (Gow Mac 750P). The detector and injection ports were maintained at 210℃ and column was maintained at 180℃, respectively. Helium was used as carrier gas at a flow rate of 30 ml/min. The integration and calibration of peak areas were carried out with an integrator (Spectra-Physics SP4290).

Whole Cell Immobilization

The immobilization of the whole cells of *Brevibacterium* sp. CH2 in polyacrylamide gel was performed by using a technique similar to that previously described (12).

Reactor Operation

A jacketed glass column, equipped with sintered glass bottom to support immobilized cells, was used. The substrate reservoir was placed in a water bath and the packed bed was maintained at the same temperature (4℃) as that of the reservoir by recirculating water from the water bath through a plastic tube. The reactor operation was carried out in the continuous type by continuously feeding the substrate solution (0.91 mol/l acrylonitrile) into the packed bed (2.0 cm bed diameter and 100 cm height).

RESULTS AND DISCUSSION

Determination of Kinetic Parameters

Lineweaver-Burk plots for the amounts of acrylamide formed are shown in Fig. 1. The Michaelis-Menten mechanism holds for the nitrile hydratase system of CH1 and CH2 strain, giving the Michaelis-Menten constants V_{max} and K_m. The substrate inhibition constants K_s of nitrile hydratase was obtained from the plot of 1/V vs. S as shown in Fig. 2. To obtain the product inhibition constants K_p of nitrile hydratase of CH1 and CH2 strain, Dixon plot in which acrylamide is an inhibitor is shown in Fig. 3. These kinetic parameters are tabulated in Table 1.

The study of the effect of acrylonitrile concentration on the reaction velocities in the absence and presence of acrylamide as inhibitor indicated a competitive type of reaction kinetics. Therefore, competitive kinetic expressions are reasonable for nitrile hydratase system.

Substrate Inhibition

Recently, to reduce acrylonitrile toxicity, we applied

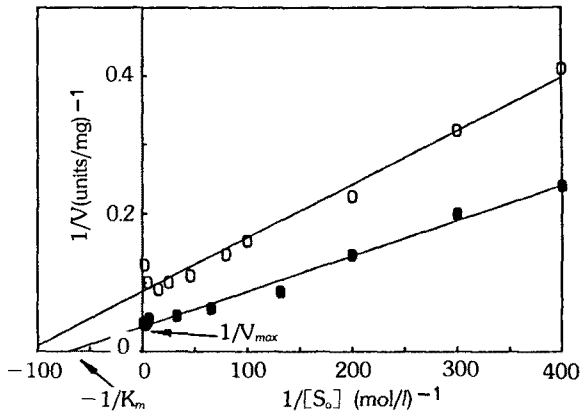

Fig. 1. Excess substrate (acrylonitrile) inhibition of nitrile hydratase of *Brevibacterium* sp. (Lineweaver-Burk plot).
(○) CH1 strain; (●) CH2 strain.

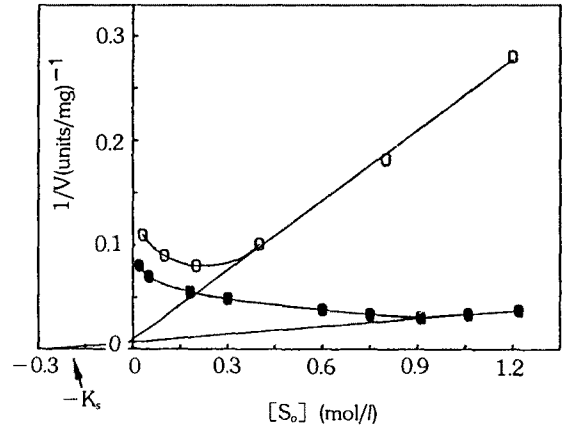

Fig. 2. K_s determination from the plot of 1/V vs. [S_o].
(○) CH1 strain; (●) CH2 strain.

the acrylonitrile adaptation that added acrylonitrile previously concentrated medium to culture broth as supplementing nutrients. As a result of this adaptatioin, *Brevibacterium* sp. CH2, having a high nitrile hydratase activity and a high acrylonitrile concentration tolerance, was developed (14).

Acrylonitrile was chosen for the substrate inhibition study since the acrylonitrile-acrylamide bioconversion is the most interesting from an industrial point of view. As shown in Fig. 2, the substrate inhibition of the nitrile hydratase activity of CH1 and CH2 strain began to appear as the acrylonitrile concentrations reached 0.2 and 0.91 mol/l, respectively. In comparison with CH1 strain, CH2 strain was greatly improved in terms of acrylonitrile tolerance.

The acrylonitrile used was very pure (above 99.5%

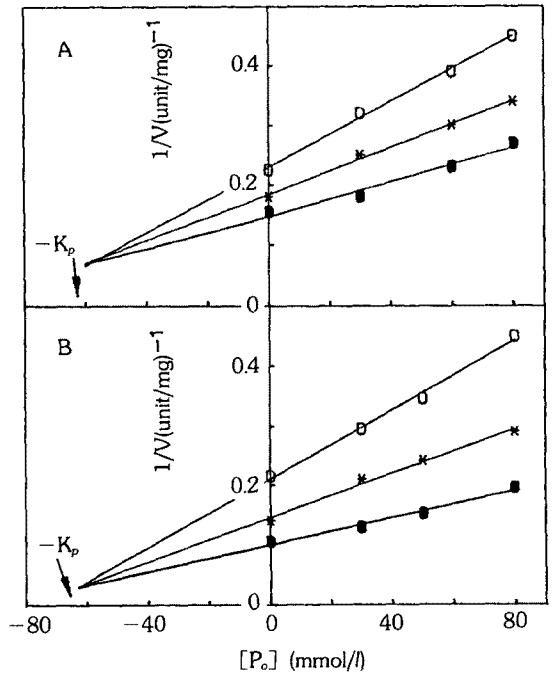

Fig. 3. Product (acrylamide) inhibition of nitrile hydratase of *Brevibacterium* sp. (Dixon plot).
(A) CH1 starin; (B) CH2 strain. (○) [S_o]=5 mmol/l; (*) 7.5 mmol/l; (●) 10 mmol/l.

Table 1. Kinetic constants used in calculations

	Brevibaterium sp. CH1	CH2
V_{max} (mol/h/g)	0.936	2.16
K_m (mol/l)	0.01	0.013
K_s (mol/l)	0.045	0.26
K_p (mol/l)	0.06	0.065

purity) and stable. We did not detect any cyanide or acrylic acids as impurity or decomposition products. The inhibition observed must be caused by an excess of acrylonitrile and not by any contamminating compound.

The substrate inhibition of previously reported strains occurred only at relatively low acrylonitrile concentration of 0.4 mol/l or less. In the case of the enzymatic production of acrylamide with any other bacterial strains, acrylonitrile was added in portions successively so as not to excess a concentration of 0.4 mol/l or less. On the other hand, acrylamide production with *Brevibacterium* sp. CH 2, using the high acrylonitrile concentration of 0.91 mol/l as substate is very feasible due to its high acrylonitrile concentration tolerance which resulted from the reduction of acrylonitrile toxicity by the acrylonitrile adaptation (14).

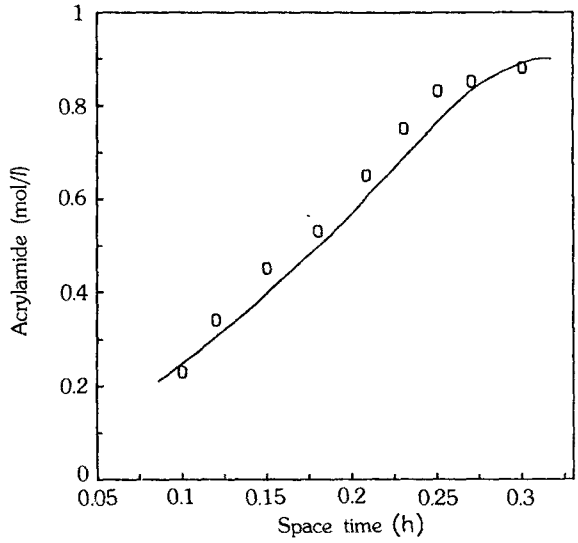

Fig. 4. Acrylamide concentration formed at different space times.
Solid line shows calculated values.

In comparison with chemical hydrolysis using high acrylonitrile concentration (1.32 mol/l) as reactant, enzymatic transformation of acrylonitrile to acrylamide could not use high concentration of acrylonitrile because of substrate inhibition. However, the biological transformation of acrylonitrile to acrylamide using *Brevibacterium* sp. CH2 is very attractive because it did not form acrylic acid as by-product and tolerated high acrylonitrile concentration (13).

Product Inhibition

Since many enzymes are inhibited by their own reaction products, we wanted to verify this for the nitrile hydratase of *Brevibacterium* sp. because the bioconversion process would become economically more attractive as the concentration of product solution, especially acrylamide, became higher.

As shown in Fig. 3, acrylamide inhibited the nitrile hydratase activity competitively. The enzyme of CH2 strain was slightly insensitive to acrylamide than that of CH1 strain. It was resulted from the adaptation of the enzyme for acrylamide formed during the acrylonitrile adaptation (14).

Analysis of Packed Bed Reactor

Fig. 4 shows a comparison between the experimental results of acrylamide production and the theoretical predictions, in which acrylamide concentration are plotted against space time in the reactor which was packed with immobilized CH2 strain. The following kinetic constants were obtained experimentally and used for the simulation: ε=0.3; η=0.4; W=5.0 g; [S_o]=0.91 mol/l; [P_o]= 0 mol/l and the values of K_m, V_{max}, K_s, and K_p are listed Table 1.

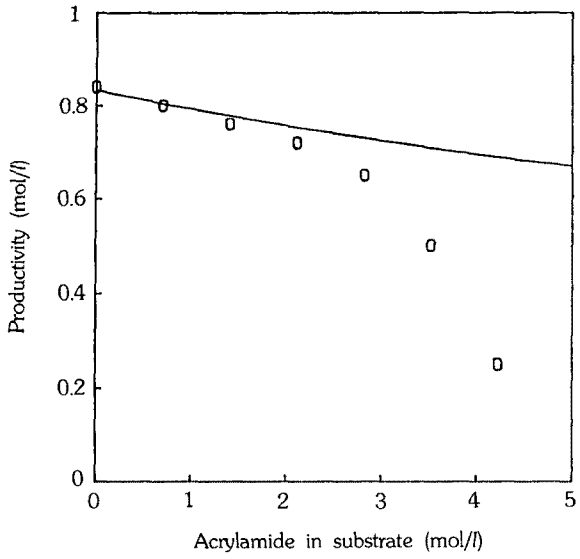

Fig. 5. Effect of acrylamide concentration on acrylamide productivity in reactor packed with immobilized whole cells of CH2 strain.
Solid line shows calculated values.

This figure shows that the acrylamide concentration of an effluent increases with space time of the reactor. If the space time increases over 0.3 h, the acrylamide concentration increases slightly and at last approaches the maximum value, which is 100% conversion point of acrylonitrile of 0.91 mol/l. The agreement between simulation and experimental results indicates the validity of dispersed plug flow model. Therefore, this analytical model can be used for designing efficient packed bed reactor systems and their scale-up, and the prediction of the best reactor performance.

Fig. 5 shows the effect of acrylamide concentration on acrylamide productivity in rector which was packed with immobilized whole cells of *Brevibacterium* sp. CH2. A feed mixture of 60 ml of acrylonitrile and 940 ml of a 0.1 M phosphate buffer aqueous solution (pH 7.0) containing acrylamide from 0 to 4.2 mol/l which corresponding to 30 (wt/v)%, respectively was introduced at a flow rate of 600 ml/h into the packed bed reactor. The agreement between simulation and experimental results at lower acrylamide concentration (below 2 mol/l which corresponding to 14.2% (wt/v)) indicates the validity of the model. However, at higher acrylamide concentration (above 2 mol/l) the experimental results deviates from those of the simulation. It might be due to the invalidity of kinetic expressions at a high product concentration. The volumetric productivity of acrylamide decreased with the increase of acrylamide concentration at 0.91 mol/l acrylonitrile concentration. When acrylamide concentration in a reactant was beyond 20% (wt/v), the productivity sharply decreased. Thus, when acrylamide is produced by the biological method, product concentration must be controlled so as not to exceed a 23% (wt/v) due to its inhibitory effect on the *Brevibacterium* sp. CH2 enzyme.

CONCLUSIONS

Inhibition of the nitrile hydratase began to appear at very low acrylonitrile concentration of 0.2 mol/l for CH1 strain. This strong inhibition by an excess of acrylonitrile makes the continuous production difficult (12). In comparison with 0.2 mol/l of CH1 strain, the substrate inhibition of CH2 strain occurred at a high acrylonitrile concentration of 0.91 mol/l. The high tolerance of CH2 strain with respect 0.91 mol/l acrylonitrile made a continuous operation feasible (13). Also the activity of the nitrile hydratase of CH2 strain was only slightly affected by acrylamide, except at a concentration higher than 23%.

Therefore, the selection of a *Barevibacterium* sp. CH2 strain with a nitrile hydratase insensitive to the acrylonitrile and acrylamide, could provide an interesting improvement in the biological production of acrylamide. Also the analysis of a packed bed reactor is useful in the preliminary design of the reactor system. Eventually, the analysis should extend to the reactors in a series with intermittent substrate feeding for a continuous acrylamide production.

Acknowledgement

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Nomenclature

$[E_o]$: total enzyme concentration
$[S_o]$: initial substrate concentration (mol/l)
$[S]$: substrate concentration (mol/l)
$[S_f]$: final substrate concentration (mol/l)
$[P_o]$: initial product concentration (mol/l)
$[P]$: product concentration (mol/l)
V_{max} : maximum reaction rate (mol/h/g)
k_1 : forward reaction rate constant
k_{-1} : backward reaction rate constant
K_m : substrate concentration where the reaction velocity is equal to $V_{max}/2$ (mol/l)
K_p : product inhibition constant (mol/l)
K_s : substrate inhibition constant (mol/l)
V : reaction rate (mol/h/g)
ε : void fraction
τ : space time (h)

u_s : superficial velocity (cm/h)
W : cell mass, dry weight (g)
L : reactor length (cm)
A : cross-sectional area of the reactor (cm^2)
Q : feed rate (ml/h)

Greek Letters

η : effectiveness factor

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