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Optimum Culture Conditions of *Brevibacterium* sp. CH2 for Production of Nitrile Hydratase

CHOI, SANG KYO, CHEO YOUNG LEE, HO NAM CHANG*, AND JUN SIK HWANG[1]

Bioprocess Engineering Research Center and Department of Chemical Engineering
Korea Advanced Institute of Science and Technology, Taeduk Science Town
Taejeon 305-701, Korea
[1]Explosive Train and Pyrotechnic Division, Agency for Defense Development
P.O. Box 35, Taejeon 305-152, Korea

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Optimum culture conditions for the formation of nitrile hydratase by *Brevibacterium* sp. CH2 were investigated. Addition of ferric and ferrous ions greatly increased the nitrile hydratase formation. The effects of nitriles, amides, and acids as an inducer on the formation of nitrile hydratase were investigated. Isobutyramide was the best inducer among the tested compounds. When *Brevibacterium* sp. CH2 was cultivated for 23 h at 30°C in a optimized medium containing 15 g of glucose, 5 g of bacto peptone, 3 g of yeast extract, 3 g of malt extract, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NaCl, 0.5 g of isobutyramide, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.02g of $FeSO_4 \cdot 7H_2O$ per liter of distilled water with pH controlled at 7.1, the maximum total activity was 665 units/ml of the culture broth and the specific activity was 70 units/mg of the dry cells. The medium optimization increased the specific activity of *Brevibacterium* sp. CH2 2.2 times.

Acrylamide is a commodity chemical which is the monomer for polyacrylamide. Polyacrylamide is additives for flocculant agents in sewage treatment, petroleum recovery, paper making, textile sizes and other industrial materials and its world demand is about 200,000 tons per year. The conventional acrylamide synthesis by copper salt catalyst uses a acrylonitrile concentration of 7% (w/v) as reactant. However, the catalyst preparation, the regeneration of the used catalyst, the separation and purification of chemically made acrylamide are laborious, and the process requires a high temperature of 90℃. Nonetheless, it is desirable to produce acrylamide at lower temperature because acrylamides are rapidly polymerized.

Galzy and co-workers, and Yamada and his associates proposed an enzymatic process for the production of acrylamide which is quite different from the chemical method. Several groups of bacteria such as *Nocardia* (9, 13, 14), *Brevibacterium* (1, 6-8, 11, 14, 18), *Arthrobacter* (3-5, 26), *Rhodococcus* (22), *Corynebacterium* (24), and *Pseudomonas* (10, 27) are known to be able to convert nitriles to corresponding amides. For the microbial transformations of nitriles, two mechanisms were suggested. One is a direct hydrolysis of nitrile to carboxylic acid and ammonia, catalyzed by nitrilase (2, 10, 12, 13, 18).

$$R-CN \xrightarrow[+2H_2O]{\text{Nitrilase}} R-COOH+NH_3$$

The other is a two-step degradation pathway of nitrile which involves nitrile hydratase and amiase, with amide as an intermediate (2, 3, 6, 18, 25, 26).

$$R-CN \xrightarrow[+H_2O]{\text{Nitrile hydratase}} R\text{-}CONH_2$$

$$\xrightarrow[+H_2O]{\text{amidase}} R-COOH+NH_3$$

Hwang and Chang (15, 16) produced acrylamide using *Brevibacterium* sp. CH1 in a recycle fed-batch reactor and in a dual hollow fiber bioreactor. *Barvibacterium* sp. CH1 also transforms acrylonitrile into acrylic acid in a two-step degradation pathway. The stratin has

*Corresponding author
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a nitrile hydratase activity for acrylonitrile, but amidase activity toward acrylamide was negligible. Therefore, the conversion yield was nearly 100% with a trace amount of acrylic acid produced.

The formation of nitrile hydratase in *Psedomononas chlororaphis* B23 (27) and *Brevibacterium* R312 was greatly enhanced by the addition of ferrous and ferric ions to the medium (21). These nitrile hydratases contain ferric ions at their active centers (23). Also, the addition of $CoCl_2$ greatly increased the nitrile hydratase activity of *Rhodococcus rhodochrous* (22).

Recently, we developed *Brevibacterium* sp. CH2 by repeated cultivation of *Brevibacterium* sp. CH1 in the broth with gradually increased acrylonitrile concentration. The specific nitrile hydratase activity of the CH2 strain was 3.2 times that of CH1 strain and the CH2 enzyme has higher acrylonitrile concentration tolerance than that of the CH1 enzyme (19, 20).

For the economical production of acrylamide the enzyme produced by the cells should have the following properties: it should have temperature stability so that commercial operation can be carried out at a temprature higher than 4℃, currently known optimum temperature for a commercial operation; it should have higher tolerance toward acrylamide and acrylonitrile; it should possess a high specific activity. The purpose of this study is to search the optimum culture conditions for *Brevibacterium* sp. CH2.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Brevibacterium sp. CH2 which was selected from *Brevibacterium* sp. CH1 as the highest acrylamide producing strain in our laboratory (20) was use in this study. In studying culture conditions, *Brevibacterium* sp. CH2 was subcultured at 30℃ for 24 h with reciprocal shaking in a 250 m*l* flask containing 50 m*l* of a basal medium containing of glucose, 15 g; yeast extract, 3 g; malt extract, 3 g; bacto peptone, 5 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; NaCl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g per liter of distilled water. The initial pH of the medium was adjusted to 7.1 with a 2 N NaOH solution.

The seed cultivation was inoculated with *Brevibacterium* sp. CH2 in a 250 m*l* Erlenmeyer flask containing 50 m*l* of the optimized medium containing of glucose, 15 g; yeast extract, 3 g; malt extract, 3 g; bacto peptone, 5 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; NaCl, 1 g; isobutyramide, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.02 g per liter of distilled water and cultivated aerobically on a rotary shaker with 300 rpm at 30℃ for 1 day. Batch fermentation was carried out in a 5 liter fermenter (KFC, Korea Fermenter Co.) at 30℃, pH 7.1, and 500 rpm agitation. Aeration was performed by bubbling filtered air into the vessel at 1 vvm.

Assay for Nitrile Hydratase Activity

Nitrile hydratase activity of the whole cells was assayed in a reaction mixture containing 1 m*l* of 6% (v/v) acrylonitrile solution and centrifuged whole cells obtained from 1 m*l* of the culture broth. Acrylonitrile solution was prepared by adding 60 m*l* acrylonitrile to 1 liter of 0.1 M potassium phosphate buffer (pH 7.0). Reaction was carried out at pH 7.0 and 5℃ for 2 min with moderate shaking and then terminated by adding 0.1 m*l* of conc. (ca. 35%) HCl. One unit of nitrile hydratase activity was defined as the amount of the whole cells that catalyzed the formation of 1μmoles of acrylamide per min under this reaction condition. The specific activity was expressed as units per mg of the dry cells.

Analytical Methods

The optical density (OD) of the cell broth was measured at 610 nm with a spectrophotometer (Milton Roy Co., Spectronic 20D). One optical unit was equivalent to 0.38 g dry wt/*l*. Glucose was colorimetrically assayed with glucose oxidase and peroxidase with a Glucose E-Kit (Yeongdong Pharm Co., Seoul).

The amount of acrylamide formed in the reaction mixture was determined by gas chromatography (GC) equipped with a flame ionized detector. GC was performed with a Varian 3300 equipped with a column packed with chromosorb W80-100 mesh as solid phase and carbowax 20 M 10% as stationary phase. The operational conitions were: detector and injection port temperature, 210℃; column temperature, 160℃. Helium was used as carrier gas at a flow rate of 30 m*l*/min. The intergration and calibration of peak areas were carried out with an intergrator (Spectra-Physics SP4290).

RESULTS AND DISCUSSION

Effects of Carbon Sources

In *Pseudomonas chlororaphis* B23 (27), when the carbon source was glucose, the presence of glucose in the culture medium seemed to inhibit the formation of nitrile hyratse. However, when the carbon source was sucrose, little glucose was detected in the culture broth. Surcrose seems to be digested gradually to glucose and fructose by sucrase, and the glucose formed is probably metabolized very quickly. Accordingly, glucose did not accumulate in the culture broth. Thus, to avoid catabolite repression, sucrose was the best carbon source.

However, as shown in Table 1, glucose was the best carbon source for the formation of nitrile hydratase of *Brevibacterium* sp. CH2. Fructose and sucrose were good, too. Maltose and molasses were not effective in promoting the growth of the cells or the formation of

Table 1. Effects of carbon sources on the formation of nitrile hydratase and the cell growth of *Brevibacterium* sp. CH2

Cabron source	OD	Specific activity (units/mg cells)	Total activity (units/ml broth)
D-glucose	11.5	32.1	140.3
D-fructose	9.5	24.6	117.0
Maltose	6.5	13.4	33.0
Sucrose	10.1	24.1	120.0
Molasses	5.6	4.3	7.9

Various carbon sources were added at final concentration of 1.5% (w/v) to the medium consisted of bacto peptone 5 g, yeast extract 3 g, malt extract 3 g, KH_2PO_4 1 g, K_2HPO_4 1 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g per 1-liter of distilled water.

Table 2. Effects of organic nitrogen sources on the formation of nitrile hydratase and the cell growth of *Brevibacterium* sp. CH2

Nitrogen source	OD	Specific activity (units/mg cells)	Total activity (units/ml broth)
Yeast extract	12.2	24.8	115.2
Bacto peptone	5.2	10.0	19.8
Casamino acid	1.8	0.8	0.5
Corn steep liquor	1.6	1.5	0.9
Urea	0.6	0.5	0.1
Malt extract	0.9	0.6	0.2
NH_4NO_3	4.2	22.1	35.3
$(NH_4)_2SO_4$	4.0	17.1	26.0
Complex*	11.5	32.1	140.3

Various nitrogen sources were added at the final concentration of 1.1% (w/v) to the medium consisted of glucose 15 g, KH_2PO_4 1 g, K_2HPO_4 1 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g per 1-liter of distilled water.

*Complex nitrogen consisted of bacto peptone 5 g, yeast extract 3 g, malt extract 3 g.

nitrile hydratase.

Effects of Nitrogen Sources

Addition of urea, $(NH_4)_2SO_4$, NH_4NO_3, malt extract, and casamino acid did not promote the cell growth or the enzyme formation (Table 2). Yeast extract was good for the cell growth, and the best nitrogen source for the formation of the enzyme and the cell growth was complex nitrogen consisted of 5 g/l bacto peptone, 3 g/l yeast extract and 3 g/l malt extract.

Effects of Inorganic Compounds on the Formation of Nitrile Hydratase by *Brevibacterium* sp. CH2

The formation of nitrile hydratase in *Pseudomonas chlororaphis* B23 (27) and *Brevibacterium* R312 (21) was highly enhanced by the addition of ferrous or ferric ions to the medium. The adition of ferrous or ferric ions was indispensable for the formation of nitrile hydratase, because this enzyme has a ferric active center to which the nitrile group of the subsrate binds (23). In the case of *Rhoococcus rhodochrous*, only $CoCl_2$ caused prominent enhancement of the nitrile hydratase activity. Thus, cobalt ion is indispensable for obtaining high nitrile hydratase activity, and the ferrous and ferric ions did not play any role in the promoting formation of nitrile hyratase in *Rhdococcus rhododhrous* J1 (22).

Table 3. Effects of inorganic compounds on the formation of nitrile hydratase and the cell gorwth of *Brevibacterium* sp. CH2

Inorganic compounds	Concentration added (mg/l)	OD	Specific activity (units/mg cells)	Total activity (units/ml broth)
None		11.5	32.1	140.3
$FeCl_3 \cdot 6H_2O$	5	10.9	50.1	207.5
	10	11.3	59.4	255.1
	20	11.2	65.2	277.5
	30	11.8	57.2	256.5
$FeSO_4 \cdot 7H_2O$	5	11.3	52.1	203.9
	10	10.8	60.5	248.3
	20	10.8	68.4	280.7
	30	10.9	58.2	241.1
$CuSO_4 \cdot 5H_2O$	10	11.2	29.1	123.6
CuCl	10	11.6	34.4	151.7
$CaCl_2 \cdot 2H_2O$	10	10.5	25.7	102.5
$BaCl_2 \cdot 2H_2O$	10	9.9	30.1	113.3
$CoSO_4 \cdot 7H_2O$	10	8.8	27.5	92.9
$CoCl_2 \cdot 6H_2O$	10	10.0	25.9	98.4
$ZnSO_4 \cdot 6H_2O$	10	10.8	34.0	139.5
$MnSO_4 \cdot 5H_2O$	10	10.8	36.7	150.8
$Al_2(SO_4)_3 \cdot 6H_2O$	10	10.6	32.2	129.7

Various inorganic compounds were added at the indicated concentration to the basal medium.

The effect of various inorganic compounds on the formation of nitrile hydratase in *Brevibacterium* sp. CH2 were examined (Table 3). The addition of $FeSO_4$ and $FeCl_3$ greatly increased the formation of nitrile hydratase of *Brevibactrium* sp. CH2, which is similar to *Pseudomonas chlororaphis* B23 (27) and *Brevibacterium* R312 (21). However, the addition of coboalt ion did not enhance the nitrile hydratase activity of the cells, which is different from *Rhdococcus rhodochrous* J1 (22). The optimum concentration of $FeSO_4 \cdot 7H_2O$ was 0.02 g/l based on the total activity. Therefore, it can be concluded that ferrous and ferrc ions are indispensable for obtaining high nitrile hydratase activity in *Brevibacterium* sp. CH2. The result suggests that nitrile hydratase in *Brevibacterium* sp. CH2 requires ferrous or ferric ions as a cofactor, similarly to the enzymes in *Psedomonas chlororaphis*

Table 4. Effects of amino acids on the formation of nitrile hydratase and the cell growth of *Brevibacterium* sp. CH2

Amino acids	OD	Specific activity (units/mg cells)	Total activity (units/ml broth)
L-Cysteine	7.0	48.6	129.3
L-Cystine	6.5	50.2	124.0
L-Glutamine	8.8	46.4	155.2
L-Methionine	8.5	45.0	145.4
L-Proline	9.4	74.0	264.3
L-Serine		no growth	
Mixture*	7.8	42.1	124.8

Various amino acids were added at the final concentration of 0.05% (w/v) to the basal medium.
*Mixture consisted of L-cysteine 0.2 g, L-methionine 0.2 g, L-proline 0.2 g.

B23 (27) and *Brevibacterium* R312 (21).

Effects of Amino Acids

We examined the effect of various amino acids on the formation of nitrile hydratase (Table 4). The addition of L-proline increased the specific activity, but decreased the cell growth. The other amino acids tested in this work did not promote the cell growth or the enzyme formation.

Effects of Nitriles, Amides, and Acids on the Formation of Nitrile Hyratase

The nitrile hydratase of *Pseudomonas chlororaphis* B23 is induced by isobytyronitrile (5). Table 5 shows that nitrile hydratase of *Brevibacterium* sp. CH2 was induced by propionitrile and isobytyronitrile, but not by acetonitrile, benzonitrile, and acrylonitrile. Isobutyronitrile was the best inducer among the nitrile compounds tested. Isobutyronitrile, being volatile, was partly lost during fermentation by aeration. Therefore, we searched for an inducer that could replace isobutyronitrile and found out that isobutyramide a catabolic metabolite of isobutyronitrile, also induced the formation of the enzyme (Table 5). The addition of isobutyramide resulted in the formation of nitrile hydratase with a higher total activity, than with isobutyronitrile. Methacrylamide and nicotinamie slightly induced the enzyme formation, but not as much as isobutyramide. The inducibility of acids tested was low.

Cell Growth and Nitrile Hydratase Production

Typical time courses of the cell growth and the acrylamide production activity of *Brevibacterium* sp. CH2 grown on the optimized medium are shown in Fig. 1. The strain grew well on the optimized medium and the activity increased with the cell growth. When glucose was completely consumed after 24 h of the cultivation, around late exponential growth phase, the cell concentration and the activity of the cells reached the maxima. More than 60 units/mg of the dry cells of specific activity was attained and the maximum cell cencentration of 9.2 g/l was obtained.

The initial pH of the cultivation was about 7.1, the pH of the medium increased slowly to 7.8 and then

Table 5. Effects of nitriles, amides, and acids on the formation of nitrile hydratase and the cell growth of *Brevibacterium* sp. CH2

Nitriles, amides, and acids	OD	Specific activity (units/mg cells)	Total activity (units/ml broth)
Acetonitrile	10.6	15.5	62.4
Acrylonitrile		no growth	
Benzonitrile		no growth	
Isobutyronitrile	10.8	73.6	302.1
Propionitrile	9.1	70.4	243.4
Acetamide	8.8	22.1	73.9
Acrylamide	2.5	10.4	9.9
Isobutyramide	11.2	68.9	308.9
Methacrylamide	10.4	55.4	218.9
Nicotinamide	11.8	44.1	197.7
Propionamide	11.5	54.1	236.4
Isobutyric acid	6.1	45.2	104.8
n-Butyric acid		no growth	

Various nitriles (2 ml), amides (2 g), and acids (2 ml) were added to the basal medium.

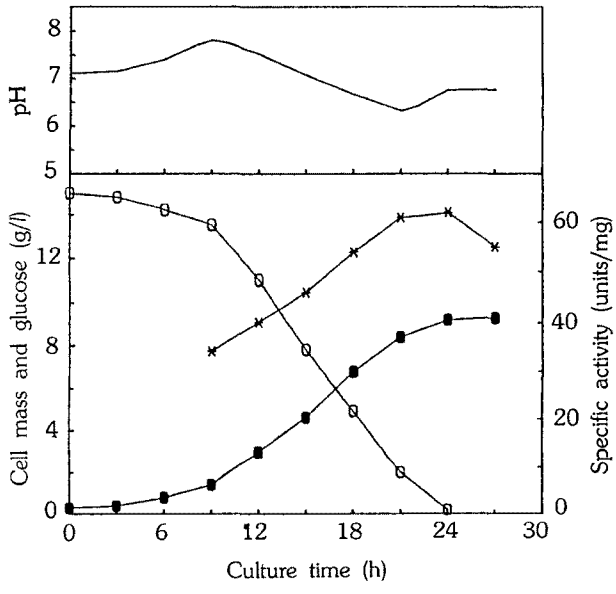

Fig. 1. Time courses of cell growth and nitrile hydratase production.
○—○: glucose concentration, ●—●: cell mass, * — *: specific activity, ——: pH; temperature: 30℃.

fell to 6.3 after 21 h of the cultivation. At about 21 h of the cultivation, the pH began to rise again. This further increase in pH coincided with the maximum levels of acrylamie bioconversion activity of the cells and the cell growth. To obtain the cells showing the highest activity, the cultivation should be stopped at this point. The same trend was found for the cultivation of *Rhodococcus* sp. N-774(24).

The effects of pH and temperature on the formation of nitrile hydratase of *Brevibacterium* sp. CH2 were previously reported (20) and the optimum pH and temperature were 7.1 and 30℃, respectively. Therefore, the pH of the broth was controlled at 7.1 during cultivation.

Fig. 2 shows the cell growth and the enzyme formation of the cells grown on the optimized medium with pH controlled at 7.1. The specific activity of the whole cells more than 70 units/mg of the dry cells was attained, and then slowly decreased during the stationary phase of the growth to remain at 60 units/mg of the dry cells. The maximum cell concentration was 10.2 g/l. Nearly all glucose was consumed after 21 h of the cultivation, around late exponential growth phase and the specific activiy and the total activity of the cells reached the maxima. The maximum total activity of 665 units/ml of the culture broth was obtained after 21 h of the cultivation and coincided with specific activity. With pH control, the cell concentration and the secific activity were increased by more 11.1% and 11.3% than without pH control, respectively.

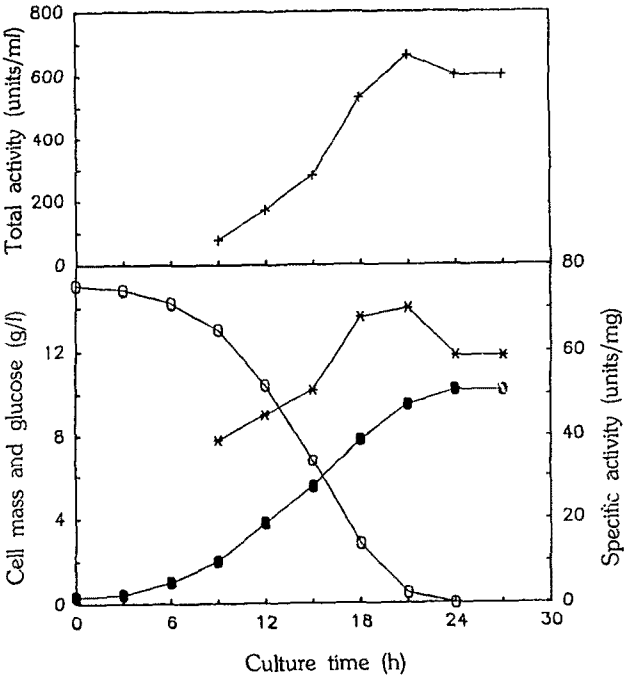

Fig. 2. Growth and activity curve in a batch cultivation with pH control.

○−○: glucose concentration, ●−●: cell mass, ＊−＊: specific activity, +−+: total activity, pH 7.1, temperature: 30 ℃.

In Fig. 3 the activities of *Brevibacterium* sp. CH1, CH2, and medium optimized CH2 are compared for various acrylonitrile concentrations. After medium optimization, the nitrile hydratase activity of the *Brevibacterium* sp. CH2 whole cells for 7% (v/v) acrylonitrile increased up to 75 units/mg of the dry cells, 2.7 times increased.

As a summary, a medium containing 15 g of glucose, 5 g of bacto peptone, 3 g of yeast extract, 3 g of malt extract, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NaCl, 0.5 g of isobutyramide, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.02 g of $FeSO_4 \cdot 7H_2O$ per liter of distilled water seems to be most suitable for the production of the cells with high nitrile hydratase activity. When *Brevibacterium* sp. CH2 was cultivated for 21 h at 30℃ with this optimum medium and pH controlled at 7.1, the nitrile hydratase productivity was high. The maximum total activity was 665 units/ml of the culture broth and the secific activity was 70 units/mg of the dry cells. The medium optimization increased the specific activity of *Brevibacterium* sp. CH2 for 6% (v/v) acrylonitrile 2.2 times. Therefore, as a first step toward the enzymatic production of acrylamide on an industrial scale, we established the suitable culture conditions for *Brevibacterium* sp. CH2.

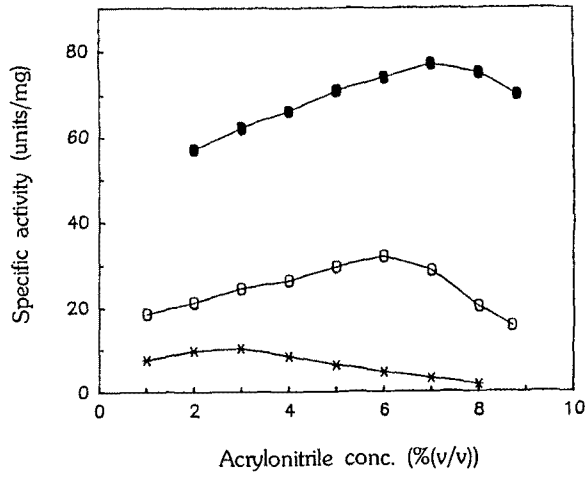

Fig. 3. Activity comparison of *Brevibacterium* sp. CH1, CH2, and medium optimized CH2 for acrylonitrile concentration.

＊−＊: CH1, ○−○: CH2, ●−●: medium optimized CH2.

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