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Isolation, Identification, and Culture Optimization of a Novel Glycinonitrile-Hydrolyzing Fungus—*Fusarium oxysporum* H3

Jin-Song Gong · Zhen-Ming Lu · Jing-Song Shi · Wen-Fang Dou · Hong-Yu Xu · Zhe-Min Zhou · Zheng-Hong Xu

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Abstract Microbial transformation of glycinonitrile into glycine by nitrile hydrolase is of considerable interest to green chemistry. A novel fungus with high nitrile hydrolase was newly isolated from soil samples and identified as *Fusarium oxysporum* H3 through 18S ribosomal DNA, 28S ribosomal DNA, and the internal transcribed spacer sequence analysis, together with morphology characteristics. After primary optimization of culture conditions including pH, temperature, carbon/nitrogen sources, inducers, and metal ions, the enzyme activity was greatly increased from 326 to 4,313 U/L. The preferred carbon/nitrogen sources, inducer, and metal ions were glucose and yeast extract, caprolactam, and Cu^{2+}, Mn^{2+}, and Fe^{2+}, respectively. The maximum enzyme formation was obtained when *F. oxysporum* H3 was cultivated at 30 °C for 54 h with the initial pH of 7.2. There is scanty report about the optimization of nitrile hydrolase production from nitrile-converting fungus.

Keywords *Fusarium oxysporum* · Glycinonitrile · Identification · Nitrile hydrolase · Optimization

Introduction

Industrial biocatalysis has been developed rapidly in recent years [1, 2]. Biocatalysis is an established method for organic synthesis, and particularly for the production of organic

J.-S. Gong · Z.-M. Zhou · Z.-H. Xu
The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, People's Republic of China

Z.-M. Lu · W.-F. Dou · H.-Y. Xu · Z.-H. Xu (✉)
Laboratory of Pharmaceutical Engineering, School of Medicine and Pharmaceutics, Jiangnan University, 1800 Lihu Road, Wuxi 214122, People's Republic of China
e-mail: zhenghxu@jiangnan.edu.cn

J.-S. Shi
Laboratory of Bioactive Products Processing Engineering, School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214122, People's Republic of China

acids such as amino acids [2–4]. It has played a more and more important role in the synthesis of amino acids from nitriles [5]. Nitrile hydrolase, mainly including nitrilase (EC 3.5.5.1) or nitrile hydratase (NHase; EC 4.2.1.84) plus amidase (EC 3.5.1.4), is the key to the hydration of nitrile to the corresponding amino acid while by-producing ammonia [6, 7]. Using enzyme preparations or whole microbial cells, hydrolysis of nitriles in organic chemical processes can be performed under mild reaction conditions with reducing wastes generation, and minimization of energy-driven and unfriendly operations when compared to the chemical synthesis [6, 8–10].

Glycine, the simplest alpha amino acid, is one of the most important commodity chemicals [11]. Its chemical functionality and unique properties make it ideal for a broad spectrum of consumer and productive applications in pesticides (for example, glyphosate), food additives, pharmaceuticals, drug intermediates, detergents, etc. [12, 13]. Conventionally, most commercial glycine has been produced by chemical processes, such as the Strecker method, in which large amounts of salts are by-produced and disposal of the by-products caused a heavy burden on the environment [13–15]. Biological methods are more acceptable because of their eco-friendly nature [16]. Furthermore, as compared with the traditional methods, it is a relatively milder synthesis and can get higher yield [10, 17]. Therefore, the application of biosynthesis is attractive for the production of glycine. So far, however, very little information reported about microorganisms, especially fungal strains harboring glycinonitrile hydrolase which can be able to produce glycine from glycinonitrile, is available though the first studies on this topic were patented as early as in the 1990s and early 2000s [14, 18, 19].

In the present study, we have attempted to isolate a novel strain capable of enzymatic converting glycinonitrile into glycine. The strain of fungus H3 isolated from soil was identified according to its morphological characteristics, and the 18S ribosomal DNA (rDNA), 28S rDNA, and the internal transcribed spacer (ITS) sequence analysis. Also, with optimization of culture conditions, a potential biocatalyst for nitrile hydrolysis was obtained and this would pave the way for the establishment of a more economical and highly productive bioprocess which could effectively transform glycinonitrile to glycine. To the best of our knowledge, there is scanty report about the optimization of nitrile hydrolase production from nitrile-converting fungus.

Materials and Methods

Chemicals and Reagents

Benzyl cyanide (98%), ε-caprolactam (98%), 3-, 4-cyanopyridine (98%), glycinonitrile (97%), glycinamide (99%), and isovaleronitrile (98%) were purchased from Sigma-Aldrich Chemical Co., Inc. (St. Louis, MO, USA); caprolactam (CP) from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); and benzonitrile (GR), 2-cyanopyridine (99%), formamide (AR), geranyl nitrile (97%), and valeronitrile (98%) from Aladdin reagent Co., Ltd. (Shanghai, China). All other reagents and chemicals used in the present study, obtained from commercial sources, were of analytical grade and used without further purification.

Cultures and Media

The nitrogen-free minimal medium used for the isolation of glycinonitrile-utilizing microorganisms contained the following (per liter of distilled water): glucose 5 g, KH_2PO_4

1 g, $MgSO_4$ 0.1 g, $FeSO_4$ 0.02 g, $CaCl_2$ 0.02 g, and NaCl 1 g. The medium was adjusted to pH 7.0 with 0.5 M NaOH or HCl solution and autoclaved using a high-pressure steam sterilizer (Tomy, SX-500, Tokyo, Japan) at 121 °C for 20 min. The solid medium was prepared by addition of 20 g agar per liter.

The rich medium (the same as the basal medium unless otherwise stated) comprised the following components per liter: glycerol 10 g, peptone 5 g, yeast extract 5 g, NaCl 1 g, KH_2PO_4 2 g, $MgSO_4$ 0.1 g, $FeSO_4$ 0.03 g, and caprolactam 20 mM, pH 7.2. The medium was autoclaved at 121 °C for 20 min. Caprolactam was used as the inducer for glycinonitrile hydrolase.

Cooked rice medium was made in 15 mm×150 mm glass tube, filled with 2 g of rice followed by addition of 6 mL distilled water, and then autoclaved at 121 °C for 30 min.

Potato dextrose agar (PDA) consisted of 200 g potato fusion, 20 g glucose, and 15 g agar per liter of distilled water, and then autoclaved at 121 °C for 30 min.

Screening and Cultivation of Glycinonitrile-Hydrolyzing Strains

Soil samples collected from different locations of Hunan Province, Jiangsu Province, and Chongqing City in China were stored in polyethylene bags and preserved at 4 °C in refrigerator. A 1:10 dilution of each sample was made with 0.9% saline solution, and the mixture was shaken intensively at room temperature for 10 min. One milliliter of the suspension was transferred into sterile blank plates and then poured into nitrogen-free mineral medium (25 mL) supplemented with 6 mM glycinonitrile as the sole nitrogen source to isolate single colonies. The medium should be cooled to 40–50 °C firstly after sterilization at 121 °C before mixing with glycinonitrile and poured into plates. The plates were then incubated at 30 °C. After 3 days of incubation, the plates were examined for colony development. The single colonies were subcultured into fresh medium containing glycinonitrile. Three to four subcultures were made to ensure the purity of new isolates before the final identification was undertaken.

The cultures for further use were prepared by inoculating the resulting isolated pure single colonies into Erlenmeyer flasks (500 mL) containing 50 mL rich medium and incubated in a flask shaker at 30 °C and 120 rpm for 48 h. The cells were harvested by centrifugation using a high-speed refrigerated centrifuge (Hitachi CR 22GII, Tokyo, Japan) under 4 °C at 12,320×*g* for 10 min and washed twice with 50 mM sodium phosphate buffer (pH 7.2), then resuspended in the same buffer and stored at 4 °C for further biotransformation.

Morphological and Molecular Identification

The fungus was conventionally identified by its morphology. Characteristics of strain H3, such as growth, colony shape, color, microconidium, and macroconidium were observed on PDA or cooked rice medium.

The present molecular identification involved 18S rDNA, 28S rDNA, and ITS sequence analysis. Chromosomal DNA of the isolate was extracted from fresh biomass cultured in PDA medium and amplification was performed with a MyGene™ Series Peltier Thermal Cycler (LongGene, Zhejiang, China) programmed for one cycle at 94 °C for 4 min, followed by 30 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 90 s. The final extension step of 72 °C for 10 min was conducted after 30 cycles. The polymerase chain reaction (PCR) was performed in volumes of 50 μL, and each reaction mixture consisted of 2 μL of DNA template, 5 μL of $MgCl_2$–10× PCR buffer, 4 μL 2.5 mM deoxynucleoside

triphosphates (dNTPs), 2 μL of 20 μM each primer, 1 μL 5 U/μL of Ex Taq DNA polymerase (Takara, Japan), and 34 μL of sterile distilled water. The gene sequences were amplified using PCR with universal primers listed in Table 1 and the PCR products were verified by the electrophoresis in a 0.8% agarose gel stained with goldview. In addition, DNA fragments were separated by size and purified using a High Pure PCR product purification kit (Invitrogen, Carlsbad, CA, USA). The resulting PCR fragments were ligated with pMD19-T vector (Takara, Japan) by T/A cloning. The constructed vectors were transformed into the competent *Escherichia coli* JM109 cells prepared by the calcium chloride-mediated method and plated onto Luria–Bertani (LB) plates containing 100 μg/mL ampicillin, 0.5 mM isopropyl β-D-thiogalactoside (IPTG), and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), incubated overnight at 37 °C for positive clone, and DNA subsequently sequenced by Invitrogen Co., Ltd. Database search was conducted with BLAST software in the National Center for Biotechnology Information (NCBI). The sequences determined and reference sequences downloaded from GenBank Database were aligned using multiple-sequence alignment software CLUSTAL X version 1.81 [24]. Phylogenetic trees were constructed with the molecular evolutionary genetics analysis software MEGA version 3.1 program (Center for Evolutionary Functional Genomics, AZ, USA) based on the partial 18S rDNA, 28S rDNA, and ITS sequences of the fungal strain H3.

Enzyme Assay

The assay was based on the release of ammonia by glycinonitrile-hydrolyzing enzymes. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of ammonia at the rate of 1 μmoL per minute under standard conditions. Specific activity was expressed as units per gram of dry cells.

The standard reaction mixture (2 mL) for glycine production contained 20 mM glycinonitrile, 50 mM sodium phosphate buffer (pH 7.2), and 0.2 mL of the cell suspension [20 g (dry weight) of cells per liter] obtained from culture broth. The reaction was carried out at 30 °C with shaking. Samples were withdrawn at regular intervals, and the reaction was terminated by centrifugation at 20,817×*g* for 5 min with a refrigerated centrifuge (Sigma 1-15K, Osterode, Germany) to remove biomass. The ammonia in the supernatant resulting from the hydrolysis was assayed spectrophotometrically (Unico UV-2100, NJ, USA) according to the phenol–hypochlorite reaction [25]. All assays were performed in triplicate.

Table 1 Primers for PCR amplification

Sequence	Primer name	Prime sequence	Reference
18S rDNA[a]	NS1	5′-GTAGTCATATGCTTGTCTC-3′	[20, 21]
	NS4	5′-CTTCCGTCAATTCCTTTAAG-3′	
	NS3	5′-GCAAGTCTGGTGCCAGCAGCC-3′	
	NS8	5′-TCCGCAGGTTCACCTACGGA-3′	
28S rDNA	LROR	5′-ACCCGCTGAACTTAAGC-3′	[22]
	LR5	5′-TCCTGAGGGAAACTTCG-3′	
ITS	ITS1	5′-TCCGTAGGTGAACCTGCGC-3′	[23]
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	

[a] 18S rDNA was amplified with two pair of universal primers, namely NS1–NS4 and NS3–NS8

Culture Conditions Optimization

Optimizations of different parameters for synthesis of glycinonitrile hydrolase by the fungal strain were carried out at different cultivation conditions and medium components. The optimum cultivation conditions were investigated by varying the initial medium pH and cultivation temperature, and effects of different medium components on biomass and enzyme activity were evaluated by addition of various carbon sources, nitrogen sources, metal ions, inducers, and these components at different concentrations.

Analytical Methods

Biomass was determined after the culture broth was collected by centrifugation at 12,320×*g* for 10 min and dried at 115 °C for 2 h.

Analysis of glycine from its transformation was performed using high performance liquid chromatography (HPLC; Agilent 1100, Santa Clara, USA). The reaction mixture was analyzed on a Hypersil AA-ODS C18, 5 μm, 250×4.6 mm column at the absorbance of 338 nm using a flow rate of 1.0 mL/min.

Results and Discussion

Isolation of Glycinonitrile-Hydrolyzing Strains

Selection on media with nitrile as the sole nitrogen source was a straightforward method for screening of nitrile-hydrolyzing microorganisms [26]. Thus, microbial enrichments in the present research were performed with glycinonitrile as the sole source of nitrogen (6 mM) and glucose (5 g/L) as a carbon source. In several previous reports, the nitrile-utilizing microorganisms were isolated or cultivated with the substrate or other nitriles as the sole source of nitrogen, carbon, and energy in the enrichment procedures [27–31]; however, in this study only much slower growth after 6 days of cultivation on the plates was observed when using glycinonitrile and some metal ions as the enrichment medium without addition of any other carbon or nitrogen sources. On the other hand, a simple and rapid colorimetric reaction for screening acetonitrile-degrading bacteria was performed by Santoshkumar et al. as reported recently [32]. The same colorimetric method was also undertaken to qualitatively confirm the glycinonitrile-hydrolyzing activities of the present microorganisms, which employed bromothymol blue (0.01%, w/v) as the pH-indicator dye. Based on the liberation of ammonia in the biotransformation reaction, a color response (from green to blue) to the increase of pH in the indicator plate was observed. This color reaction indicated that the strains responsible for color change were capable of hydrolyzing glycinonitrile with the release of ammonia.

One fungal strain marked as H3 capable of utilizing glycinonitrile as sole nitrogen source and displayed higher glycinonitrile hydrolase activity was finally chosen for further research. At a preliminary experiment, the biomass of strain H3 after 48 h of cultivation was roughly 2.5 g dry cell weight (DCW) per liter of culture broth, and the specific activity of glycinonitrile hydrolase reached 132 U/g DCW while total activity in culture broth was 326 U/L.

Morphological Characters of Fungus H3

Colony morphology of fungi may vary significantly on different media, and therefore descriptions for fungus H3 in the present study are based upon the growth conditions on

PDA plates and cooked rice medium at 30 °C (Fig. 1). The mycelia of fungus H3 in PDA are white and flocculated with the colony of light violet color. The diameter of the colony reached 49 mm at 30 °C for 4 days (inoculation with a punch of 5 mm diameter). The stroma is light pink in cooked rice medium. The macroconidia are short, falcate to almost straight, thin-walled, and without septa. Microconidia are abundant, mostly non-septate, ellipsoidal to cylindrical, slightly curved or straight, occurring in false heads (a collection of conidia at the tip of the phialide) from short monophialides.

Phylogenetic Analysis of *F. oxysporum* H3

The 18S rDNA (1,768 bp), 28S rDNA (925 bp), and ITS (558 bp) sequences of the fungus H3 were determined and compared with the NCBI data, respectively. BLAST results for 18S rDNA indicated that fungus H3 had a rather high sequence identity with strains belonging to the genera of *Fusarium* (AB110910, 99% of similarity), *Fusarium* (GQ166777, 99%), *Cordyceps* (AB067700, 99%), *Gibberella* (AB237662, 99%), and *Gibberella* (AB250414, 99%). Subsequently, this strain was found to be highly similar to a *Fusarium* strain (EF590327, 99%) through the analysis of 28S rDNA sequence. In terms of the analysis results for 18S rDNA, and 28S rDNA sequences, strain H3 was identified to be one of the fungal strains in *Fusarium* genus. Finally, comparative ITS sequence analysis also supported an extremely close relationship between fungus H3 and members of the genus *Fusarium*. Particularly, strain H3 had 100% sequence similarity with *F. oxysporum* (DQ002550). It could also be observed that the strain H3 was located in the same clade with *F. oxysporum* from the phylogenetic tree based on ITS sequence from 13 aligned sequences (Fig. 2). Therefore, by comparing its 18S rDNA, 28S rDNA, and ITS sequences, together with examining the morphological characteristics, the fungus H3 was identified to be a strain of *F. oxysporum* and named *F. oxysporum* H3.

Effects of Carbon Sources on Enzyme Activity

In some cases, enzyme overproduction through altering the culture conditions has more advantages than genetic manipulation [33]. Therefore, optimization of culture conditions was carried out by “one-variable-at-a-time” method [34]. In general, the carbon source has a conspicuous effect on enzyme activity. Various carbon sources, at a final concentration of 10 g/L, were tested for their effects on biomass and specific nitrile hydrolase activity in *F.*

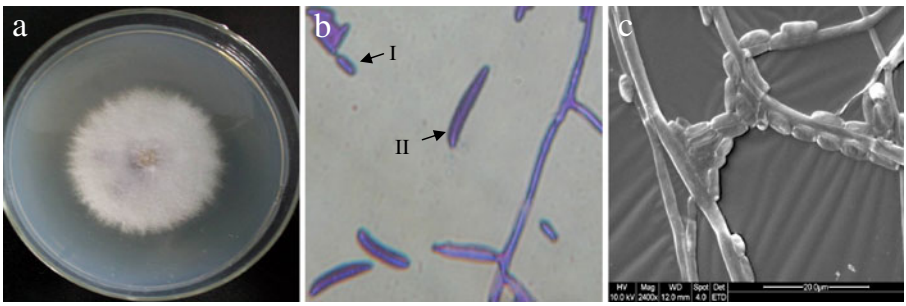

Fig. 1 Morphological characters of fungus H3. **a** Growth on PDA plate. The colony was cultivated at 30 °C for 4 days. **b** Microscopic image (×1,600). *I* microconidium, *II* macroconidium. **c** Scan electron micrograph (×2,400)

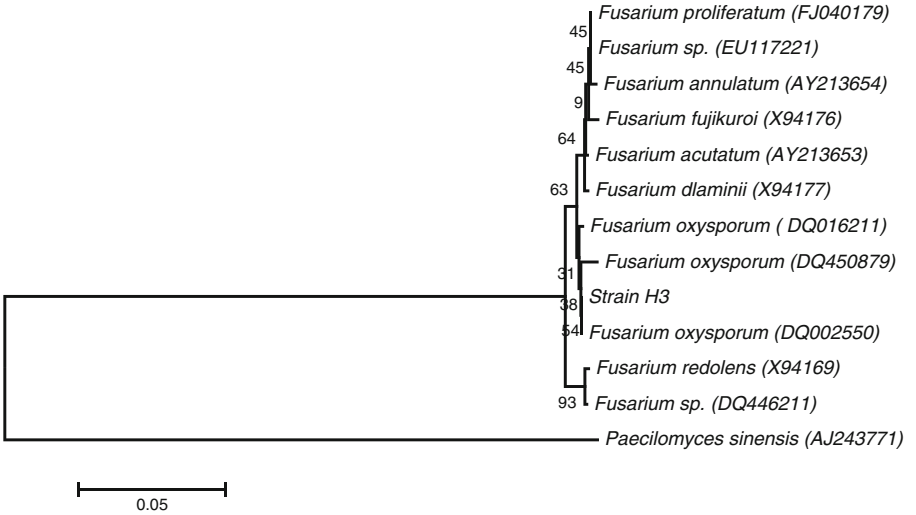

Fig. 2 Phylogenetic tree indicating relationship among isolates of *Fusarium* based on ITS sequences, constructed by the neighbor-joining method. *Numbers* in *parentheses* are accession numbers of published sequences in GenBank. Bootstrap values were based on 1,000 replicates. *Paecilomyces sinensis* was used as the outgroup

oxysporum H3 when 5 g/L peptone and 5 g/L yeast extract were used as the nitrogen source and the culture time was 60 h. As shown in the results in Table 2, the addition of soluble starch, glycerol, fructose, glucose, sucrose, and mannitol enhanced both the strain growth and relative enzyme activity. However, ammonium acetate and sodium glutamate did not support the enzyme activity as well as growth of *F. oxysporum* H3. On the other hand, the organic acids, such as succinic acid and fumaric acid, did not serve as excellent

Table 2 Effects of carbon sources on biomass and formation of glycinonitrile hydrolase

Carbon source	Biomass (g/L culture broth)	Relative enzyme activity[a] (%)
None	2.60±0.50	9.64±0.94
Ammonium acetate	2.25±0.55	ND
Succinic acid	2.60±0.70	2.16±0.93
Corn flour	4.37±0.71	9.31±1.22
Sodium glutamate	3.37±0.15	9.98±0.61
Benzoic acid	3.83±0.43	12.77±1.52
Fumaric acid	2.93±0.23	13.38±0.30
Lactic acid	3.00±0.90	13.72±1.25
Sodium citrate	2.97±0.43	24.60±2.16
Soluble starch	4.97±0.89	20.23±1.55
Acetonitrile	2.00±0.35	36.47±0.94
Glycerol	2.47±0.49	48.29±2.79
Fructose	6.93±0.33	61.25±2.31
Glucose	8.07±0.95	79.44±0.30
Sucrose	7.27±0.77	88.47±1.86
Mannitol	4.83±0.59	100.00±2.19

F. oxysporum H3 was cultivated with 5 g/L peptone and 5 g/L yeast extract as the nitrogen source and the culture time was 60 h

ND not detected

[a] The relative activity was expressed as the percentage of the specific activity using mannitol as the carbon source

growth substrates. It is of interest that the expression of glycinonitrile hydrolase was not repressed by carbon sources such as glycerol and glucose resulting from catabolite repression, as described previously for bacteria [7]. In terms of enzyme activity, biomass, and cost for industrial application, glucose was selected as the most suitable carbon source. Subsequently, the effect of glucose concentration (values ranged from 5 to 20 g/L) on growth and enzyme activity was investigated. Low concentration of glucose gave higher specific activity but obtained poor biomass while the opposite result was obtained at a high concentration. Thus, 10 g/L glucose as carbon source was suitable for cultivating the fungal strain.

Effects of Nitrogen Sources on Enzyme Activity

Nitrogen source is one of the most important medium ingredients for the growth of microorganisms. Various nitrogen sources, both organic and inorganic, at the indicated concentrations were examined for their effects on enzyme activity in *F. oxysporum* H3 when 10 g/L glucose was used as the carbon source and the culture time was 60 h (Table 3). Organic nitrogen sources, such as peptone, beef extract, and yeast extract, were proved to be more suitable for glycinonitrile hydrolase expression and the fungus growth compared to inorganic sources. On the other hand, ammonium thiocyanate strongly inhibited the enzyme activity of *F. oxysporum* H3, and urea, a common nitrogen source, repressed the growth of the isolate. Acetonitrile and the corresponding amide were both ineffective for the enzyme activity but increased the cell mass production slightly. Furthermore, effects of the combination of peptone plus yeast extract and peptone plus beef extract were determined, and initial data obtained from a series of experiments

Table 3 Effects of nitrogen sources on cell growth of *F.* ox*ysporum* H3 and relative enzyme activity

Nitrogen source (g/L)	Biomass (g/L culture broth)	Relative enzyme activity[a] (%)
None	1.30±0.61	20.17±0.20
Ammonium thiocyanate 5	2.75±0.75	4.14±0.39
Urea 5	0.40±0.10	8.58±1.02
Acetamide 5	1.90±0.71	11.17±0.57
Acetonitrile 5	2.03±0.55	14.54±1.56
Ammonium sulfate 5	2.00±0.90	15.93±2.35
Peptone 5	5.63±0.31	41.18±0.39
Beef extract 5	4.40±0.14	45.24±2.82
Yeast extract 5	8.30±0.10	100.00±4.06
Peptone 20 plus beef extract 10	15.15±2.46	20.26±3.78
Peptone 10 plus beef extract 5	7.47±0.17	57.38±3.39
Peptone 2.5 plus beef extract 2.5	5.50±0.26	66.16±7.92
Peptone 20 plus yeast extract 10	20.53±1.06	8.34±0.41
Peptone 10 plus yeast extract 10	20.30±1.05	18.38±3.78
Peptone 10 plus yeast extract 5	12.43±0.72	28.40±0.99
Peptone 2.5 plus yeast extract 2.5	7.30±0.44	83.69±4.67

F. oxysporum H3 was cultivated with 10 g/L glucose as the carbon source and the culture time was 60 h

[a] The relative activity was expressed as the percentage of the specific activity using yeast extract as the nitrogen source

differing sources concentrations indicated that the combination nitrogen source favored heavy biomass, especially using the composite of 20 g/L peptone plus 10 g/L yeast extract which strongly support biomass generation (as shown in Table 3), an enhancement of more than 15-fold compared to the none counterpart. However, the source of high concentrations sharply repressed expression of the nitrile hydrolase. As a result, yeast extract was finally chosen for further researches as it exhibited higher enzyme activity as well as supported abundant biomass. In addition, 7.5 g/L of yeast extract (different values ranged from 5 to 20 g/L) was found to be the most favorable for the glycinonitrile hydrolase activity.

Effects of Inducers on Enzyme Activity

To our knowledge, most of the nitrile-converting enzymes are inducible by certain nitriles, amides, or their analogs, and there are little data on constitutive enzymes besides nitrilase from *Bacillus subtilis* ZJB-063 [35] and that of *Alcaligenes* sp. ECU0401 [36] as well as multiple enzymes including nitrilase and NHase/amidase in *Amycolatopsis* sp. IITR215 [37]. Induction of enzyme activity, as the cell's physiology, plays an important role in the biocatalytic processes [1]. Enzyme activity was induced when *F. oxysporum* H3 was cultured with caprolactam. Cells cultivated in the absence of caprolactam did not have this enzyme activity. Thus, various compounds (final concentration 20 mM), such as nitriles, amides, amines, carboxylic acids, and so on, were tested for their ability to induce the enzyme synthesis when 10 g/L glucose was used as the carbon source and 7.5 g/L yeast extract as the nitrogen source and the culture time was 60 h. As shown in Table 4, glycinonitrile hydrolase was effectively induced by iminodiacetonitrile, but cell growth was strongly inhibited, while benzonitrile, benzyl cyanide, 4-cyanopyridine, and isovaleronitrile almost completely did not induce the formation of the enzyme although they gave good growth. Glycinonitrile is a substrate of the enzyme, but it has only an insignificant improvement on the enzyme activity. 2-Cyanopyridine, which was regarded as a universal inducer in filamentous fungi such as strains belonging to the genera of *Aspergillus*, *Fusarium*, and *Penicillium* [38], however, could not effectively act on the enzyme production of the present fungal strain. In addition, we examined the effects of amide and amine compounds on the nitrile hydrolase. Benzamide, *N*,*N*-dimethylformamide, and formamide slightly induced the enzyme formation, as shown in Table 4. The iodoacetamide, famous as a cysteine protease inhibitor, showed a strong inhibitory property during cell cultivation of *F. oxysporum* H3. The inhibition for the enzyme by amine compounds, such as diethylamine and aniline, was also observed. As one of the best known inducers with great potential for nitrile-hydrolyzing enzyme induction [39], caprolactam was the most efficient inducer and could enhance the enzyme formation significantly. Furthermore, in present studies, we noted that caprolactam obtained from Sinopharm Chemical Reagent Co., Ltd. favored heavier biomass and higher enzyme activity than ε-caprolactam from Sigma-Aldrich Chemical Co., Inc. Diacetyl is a natural byproduct of yeast fermentation, and it led to the most excellent cell growth in the compounds tested but severely repressed the enzyme formation. The acids such as glycine and benzonic acid did not act as the effective inducers, although they supported abundant biomass. No more suitable inducers other than caprolactam were found among these putative compounds tested, and therefore 30 mM caprolactam (from 5 to 40 mM) was selected as the optimum inducer for further research. So far, the induction mechanism of nitrilase in filamentous fungi has not yet been fully demonstrated.

Table 4 Effects of nitriles, amides, amines, and acids on the formation of nitrile hydrolase

Inducers	Biomass (g/L culture broth)	Relative enzyme activity[a] (%)
Benzonitrile	12.87±0.31	ND
Benzyl cyanide	11.23±0.59	ND
4-Cyanopyridine	10.40±0.85	ND
Isovaleronitrile	9.80±0.80	ND
Acetonitrile	8.73±0.70	10.44±0.87
Geranyl nitrile	4.00±0.57	14.41±1.11
3-Cyanopyridine	8.33±0.70	16.55±4.06
Glycinonitrile	6.80±0.57	18.82±0.54
Valeronitrile	6.50±0.71	24.82±2.87
2-Cyanopyridine	9.13±0.73	40.45±3.89
Iminodiacetonitrile	1.80±0.28	96.32±3.68
Iodoacetamide	No growth	
Nicotinamide	9.20±0.80	ND
Acrylamide	8.93±0.32	ND
Glycinamide	8.70±1.91	ND
Acetamide	9.20±0.06	ND
Benzamide	4.13±0.53	37.75±2.16
Formamide	5.10±0.42	45.21±8.83
N,*N*-Dimethylformamide	9.87±0.95	52.62±6.43
ε-Caprolactam	8.60±0.28	88.10±1.68
Caprolactam	9.20±1.20	100.00±1.95
Diacetyl	15.75±1.18	ND
Diethylamine	7.87±0.73	ND
Aniline	3.93±0.11	9.03±0.87
Glycine	9.47±0.51	ND
Benzonic acid	13.60±0.53	25.20±1.95

F. oxysporum H3 was cultivated with 10 g/L glucose as the carbon source and 7.5 g/L yeast extract as the nitrogen source, and the culture time was 60 h

ND not detected

[a] The relative activity was expressed as the percentage of the specific activity using caprolactam as the inducer

Effects of Metal Ions on Enzyme Activity

The effects of various metal ions on the nitrile hydrolase activity in the culture were investigated. Various mineral compounds, including $CaCl_2$, $BaCO_3$, $CoCl_2{\cdot}6H_2O$, $LiSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $MnSO_4{\cdot}H_2O$, $C_4H_6O_4Pb{\cdot}3H_2O$, $FeSO_4{\cdot}7H_2O$, $FeCl_3{\cdot}6H_2O$, $NiSO_4{\cdot}6H_2O$, $Na_2MoO_4{\cdot}2H_2O$, $LaCl_3{\cdot}7H_2O$, $Al(NO_3)_3{\cdot}3H_2O$, $K_4Fe(CN)_6{\cdot}3H_2O$, $CsNO_3$, RbCl, and $AgNO_3$ were separately added at the final concentration of 0.1 mM to the medium when 10 g/L glucose was used as the carbon source, 7.5 g/L yeast extract as the nitrogen source, and 30 mM caprolactam as the inducer, and the culture time was 60 h. As shown in Table 5, the addition of $FeSO_4$ and $MnSO_4$ greatly increased both cell growth and enzyme formation. Unexpectedly, the addition of $CuSO_4$, which caused strong inhibition for biomass production of *Rhodococcus rhodochrous* J1 [40], however, resulted in the highest enhancement of enzyme generation by *F. oxysporum* H3 among the mineral compounds tested but slight decrease in cell growth. Such a positive effect on the enzyme expression was also observed in glycolonitrilase formation in *Alcaligenes* sp. ECU0401 [36]. $CsNO_3$ and RbCl were first examined for their effects on nitrile hydrolase; however, in the present study, it was proved that both of them

Table 5 Effects of metal ions on biomass and enzyme activity by *F. oxysporum* H3

Metal ions	Biomass (g/L culture broth)	Relative enzyme activity[a] (%)
None	9.95±0.07	35.54±3.09
$NiSO_4·6H_2O$	5.40±0.10	5.28±0.32
$CsNO_3$	9.85±1.44	19.46±1.64
RbCl	11.13±0.32	20.14±0.99
$LaCl_3·7H_2O$	10.00±0.79	21.13±0.64
$Al(NO_3)_3·3H_2O$	8.27±0.61	22.55±2.51
$K_4Fe(CN)_6·3H_2O$	9.57±0.06	23.26±0.16
$BaCO_3$	8.87±0.85	30.80±0.87
$CoCl_2·6H_2O$	9.87±0.97	31.57±1.22
$Na_2MoO_4·2H_2O$	8.63±0.72	32.35±3.29
$FeCl_3·6H_2O$	10.10±0.95	32.35±0.99
$AgNO_3$	12.15±0.35	33.33±0.98
$CaCl_2$	9.15±0.64	38.63±0.99
$ZnSO_4·7H_2O$	12.10±0.78	38.95±0.98
$LiSO_4·H_2O$	9.55±0.87	42.56±1.99
$C_4H_6O_4Pb·3H_2O$	8.50±0.78	47.16±1.64
$FeSO_4·7H_2O$	10.23±0.15	59.86±1.45
$MnSO_4·H_2O$	9.50±0.89	69.07±2.51
$CuSO_4·5H_2O$	8.83±0.42	100.00±0.99

F. oxysporum H3 was cultivated with 10 g/L glucose as the carbon source, 7.5 g/L yeast extract as the nitrogen source, and 30 mM caprolactam as the inducer, and the culture time was 60 h

[a] The relative activity was expressed as the percentage of the specific activity using $CuSO_4$ as the metal ion

supported higher biomass but decreased the enzyme activity. $LaCl_3$ did not favor the glycinonitrile hydrolase formation in *F. oxysporum* H3 while La^{2+} was proved to be an effective substance for the expression of *Acinetobacter* sp. AK226 nitrilase [41]. $CoCl_2$, which is essential for NHase expression as the cofactor of cobalt-containing NHase but make a strong inhibitory effect on nitrilase formation in *R. rhodochrous* J1 [40, 42], was not an effective compound for nitrile hydrolase production in *F. oxysporum* H3. $AgNO_3$, a potential nitrile-converting enzyme inhibitor in several previous reports [10, 43], strongly inhibited the nitrile hydrolase. Also, $NiSO_4$ promoted neither cell growth nor enzyme activity markedly. NaCl, KH_2PO_4, and $MgSO_4$ were necessary for growth of the organism (data not shown) and were then added to the medium. Finally, 2.72 g/L KH_2PO_4, 1.16 g/L NaCl, 0.12 g/L $MgSO_4$, 0.025 g/L $CuSO_4·5H_2O$, 0.014 g/L $FeSO_4·7H_2O$, and 0.085 g/L $MnSO_4·H_2O$ were included in the optimized medium.

Effects of Temperature and Initial pH on Enzyme Activity

Since the temperature is one of the most crucial factors for enzyme formation in the culture broth, effect of culture temperature (25, 30, and 37 °C) on specific enzyme activity was investigated. Maximum enzyme activity was observed at 30 °C (data not shown) with the initial pH of 7.2. However, the specific activity was so strongly impressed by the temperature of 25 and 37 °C that almost no enzyme activity was detected.

On the other hand, the initial pH of the media also has a great impact on nitrile hydrolase activity production. The optimization studies were carried out to examine the effect of initial pH of the medium on enzyme activity. *F. oxysporum* H3 exhibited excellent nitrile-converting activity within a broad pH range of 6.2–8.2. Maximum

enzyme production was obtained when the fungus was cultured at a medium with pH adjusted to 7.2, although the highest biomass was attained at pH 6.8, slightly higher than those at any other pH. Thus, it was clarified that the initial pH variations during fermentation periods led to negligible differences on enzyme activity except an inhibitory effect from pH above 8.2 and below 6.2.

Furthermore, optimization of culture temperature and initial pH revealed a unique phenomenon. At the temperature of 25 and 37 °C, the specific activity still remained in relative high level in the initial pH of 6.2 while it was strongly impressed under pH 7.2. It is suggested that the lower initial pH of the culture medium favored a higher stability of the nitrile hydrolase in *F. oxysporum* H3.

Time Course of Nitrile Hydrolase Production by *F. oxysporum* H3

With the purpose of achieving the maximal production of nitrile hydrolase, the growth pattern for *F. oxysporum* H3 was studied under the optimal conditions. A series of Erlenmeyer flasks were inoculated with fresh culture and removed at different culture time intervals for determining process parameters, such as biomass, enzyme activity, and pH. *F. oxysporum* H3 was grown in the optimum conditions at 30 °C for 78 h. As shown in Fig. 3, the growth rate increased markedly with the increase of incubation time up to 24 h and reached the top at this point. The glycinonitrile hydrolase activity gradually increased after 18 h and reached its maximum at 54 h reaching up to 455 U/g DCW (total enzyme activity of culture broth at 4,313 U/L), and then it decreased slowly. The decrease in biomass and enzyme activity may be attributed to fungal autolysis and cell death processes. It may be concluded that *F. oxysporum* H3 is a promising nitrile hydrolase-producing strain with great potential for glycine formation from glycinonitrile.

Conclusions

In this study, a filamentous fungus H3 was screened from soil with glycinonitrile as the sole nitrogen source and proved to harbor excellent nitrile hydrolase activity. And it was

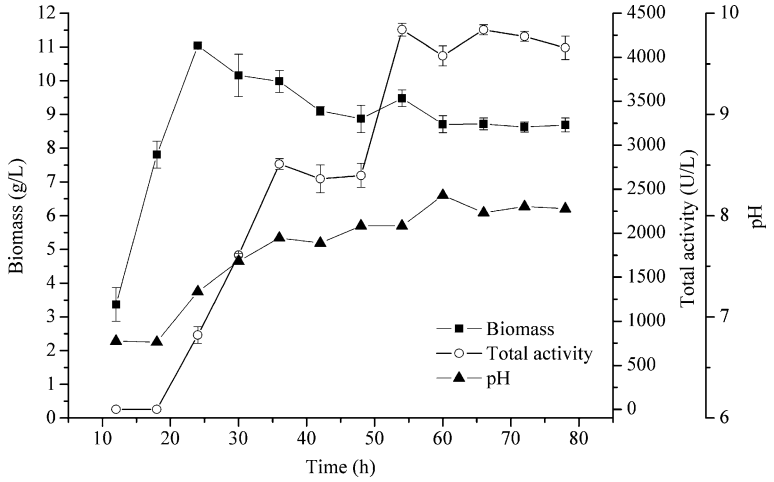

Fig. 3 Time course of biomass and nitrile hydrolase formation in *F. oxysporum* H3

identified as *F. oxysporum* according to its morphological properties and the analysis for its 18S rDNA, 28S rDNA as well as ITS sequence. An identification procedure for nitrile hydrolase-producing fungus was established in the present research. According to the further optimization of growth conditions, the nitrile hydrolase activity produced by *F. oxysporum* H3 reached up to 4,313 U/L. To date, there has been a few works focusing on nitrile hydrolase from filamentous fungi; therefore, it is of great significance to carry out the biocatalysis process mediated by fungal nitrile hydrolase. Also, the results obtained in the present investigations may indicate that this nitrile-hydrolyzing fungus has great potential for scientific and industrial application of glycine production. After sequential engineering, this fungus might be a suitable biocatalyst for glycine production at the commercial scale. Moreover, these data would lay the foundation for future research on the utilization of this strain for carboxylic acids production. The exact pathway and mechanism of biotransformation reaction for glycine from glycinonitrile by *F. oxysporum* H3 are undergoing in our laboratory.

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