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Bioconversion of Acrylonitrile to Acrylamide Using a Thermostable Nitrile Hydratase

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Abstract

Although providing an attractive route for production of acrylamide from acrylonitrile, utilization of nitrile hydratase enzymes has been limited by the requirement for low temperature bioconversion conditions. This report summarizes a search for thermostable nitrile hydratases from aerobic moderate thermophiles screened for ability to grow on acrylonitrile at concentrations to 1% at elevated temperatures. A new isolate *Bacillus* sp. BR449 constitutively expresses a thermostable nitrile hydratase with properties including low substrate inhibition and broad temperature range with optimal activity at 55°C. With prolonged exposure, BR449 nitrile hydratase exhibited temperature-dependent inactivation by acrylonitrile, which is attributed to alkylation of nucleophilic sites on the enzyme.

Index Entries: Nitrile hydratase; thermophile; acrylamide.

Introduction

Enzymatic formation of acrylamide from acrylonitrile offers advantages over the traditional copper-catalyzed process in reduction of unwanted waste products, making the process a promising example of utilization of enzymes for development of "green" processes for commodity chemical production *(1)*. Hydratases capable of catalyzing nitrile hydration have been found in a wide variety of bacteria *(2,3)*, and Nitto Chemical Company (Yokohama, Japan) has pioneered the utilization of the nitrile hydratase from *Rhodococcus rhodochrous* J1, a novel enzyme that contains cobalt ion, providing more attractive catalytic attributes than ferric ion-containing nitrile hydratases used earlier *(4)*.

Although the Nitto acrylamide process is in successful production, the bioconversion is restricted to low-temperature operation owing to inactivation of the hydratase enzyme catalyst by the acrylonitrile substrate

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and acrylamide polymerization, both of which are accelerated at higher temperatures *(4)*.

Although the determinants of enzyme stability are becoming increasingly understood *(5)*, the relationships between enzyme thermostability and resistance to chemical inactivation have been less well-investigated. It is generally recognized that at the same temperature, thermostable enzymes have less flexibility than their thermolabile counterparts, and are therefore more resistant to chemical denaturants *(6)*. Studies of spontaneous deamination, a source of enzyme inactivation at both moderate and high temperatures, have demonstrated the important influences of conformational stability and neighboring residues on rates of inactivation (reviewed in *7*).

We have initiated a project to discover nitrile hydratases from thermophile sources in an attempt to identify enzyme catalysts with improved resistance to acrylonitrile inactivation. Use of an increased temperature could provide advantages arising from increased reaction rates, higher solubility of the acrylamide product, and decreased cooling costs for this exothermic reaction. In this report, we describe the isolation and properties of a moderately thermophilic *Bacillus* isolate that produces a thermostable nitrile hydratase, and describe initial results with the enzyme in conversion of acrylonitrile to acrylamide.

Materials and Methods

Isolation of Acrylonitrile-Degrading Thermophiles

Nitrile-degrading thermophilic bacteria were isolated from acrylonitrile enrichments of soil samples collected from pristine and polluted Michigan locations and incubated at 60°C. The enrichment medium (DP) was designed to provide nutrients for more fastidious organisms, but to select for organisms capable of growth using acrylonitrile as a principal carbon source. The DP medium at pH 7.2 contained per L: K_2HPO_4, 0.5 g; NH_4Cl, 1 g; $MgSO_4$, 20 mg; yeast extract, 0.2 g; casamino acids, 0.1 g; trace element solution, 1 mL *(8)*, and acrylonitrile as desired. Primary isolations were obtained by spreading week-old suspensions of enrichment cultures on DP plates containing 0.2% acrylonitrile followed by incubation at 60°C in sealed plates. Restreaking of isolates on DP plates containing progressively higher acrylonitrile concentrations yielded thermophiles capable of growth on acrylonitrile at concentrations up to 1%.

Growth of Bacillus *Isolates*

For cells used in nitrile hydratase studies, *Bacillus* sp. BR449 was grown in OP medium that contained per L: K_2HPO_4, 1 g; KH_2PO_4, 0.5 g; yeast extract, 1 g; malt extract, 1 g; peptone 2 g; glycerol, 3 g at pH 7.2. Cobalt chloride was added to the growth medium at a concentration of 20 mg/L unless otherwise indicated. The isolate was grown at 60°C for approxi-

mately 18 h with shaking to reach late exponential phase, using turbidity to monitor growth.

Nitrile Hydratase and Amidase Assay

Nitrile hydratase activity was measured using whole cells in assays containing 20 mg (dry wt) cells, in 1 mL 0.05 *M* potassium phosphate buffer, containing 0.5 *M* acrylonitrile, pH 7.5. The reaction mixture was incubated in a water bath at 50°C with shaking for 10 min, and the reaction stopped by addition of 0.2 mL 2 *N* HCl. Acrylamide formation was measured using high-performance liquid chromatography (HPLC) by injection into a Novapak C-18 reverse phase column and eluted with 1:12 acetonitrile:5 m*M* potassium phosphate buffer, pH 2.5. Peaks were identified at 200 nm with a Waters variable wavelength detector and analyzed with a Waters 746 data module with reference to chemical standards. One unit of nitrile hydratase activity is defined as the formation of one micromole acrylamide/min. Amidase activity was measured in the same manner using 0.5 *M* acrylamide as substrate by measurement of acrylic-acid formation.

Enzyme Stability Studies

For studies of nitrile hydratase stability, whole cells of BR449 were incubated at varied temperatures for 2 h in 0.05 *M* potassium phosphate buffer, pH 7.5. The subsequent nitrile hydratase assay was carried out at 50°C.

Thermophile Identification

Thermophile isolate BR449 was identified by 16S ribosomal subunit gene sequence using universal primers and PCR methods described by Maltseva et al. *(9)*. Amplified DNA product was sequenced at the Michigan State University Sequencing Facility using an Applied Biosystems Model 173A automatic sequencer. Partial sequences were compared to Gene Bank data using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information and the Ribosomal Database Project.

Results

Characteristics of Thermophile Isolates

Following acrylonitrile enrichment of soil samples collected from varied locations, 50 isolates were selected for their growth ability on 0.2% acrylonitrile at 60°C. Following repeated transfers using increasing acrylonitrile concentration, six isolates proved capable of producing colonies on DP plates containing 1% acrylonitrile within 1 wk at 60°C. Although similar in appearance and colony morphology, these isolates demonstrated significant differences in nitrile hydratase and amidase expression (Table 1). Of these, isolate BR449 demonstrated the highest nitrile hydratase activity with only modest amidase activity, and was selected for detailed study.

Table 1
Activities of Nitrile-Degrading Enzymes in the New Isolates

New organisms	Specific activity (U/mg)	
	Nitrile hydratase	Amidase
BR443	3.0	21.4
BR444	47.2	15.5
BR445	19.2	17.5
BR446	24.8	4.9
BR447	1.1	7.0
BR448	0.8	14.6
BR449	77.6	11.3

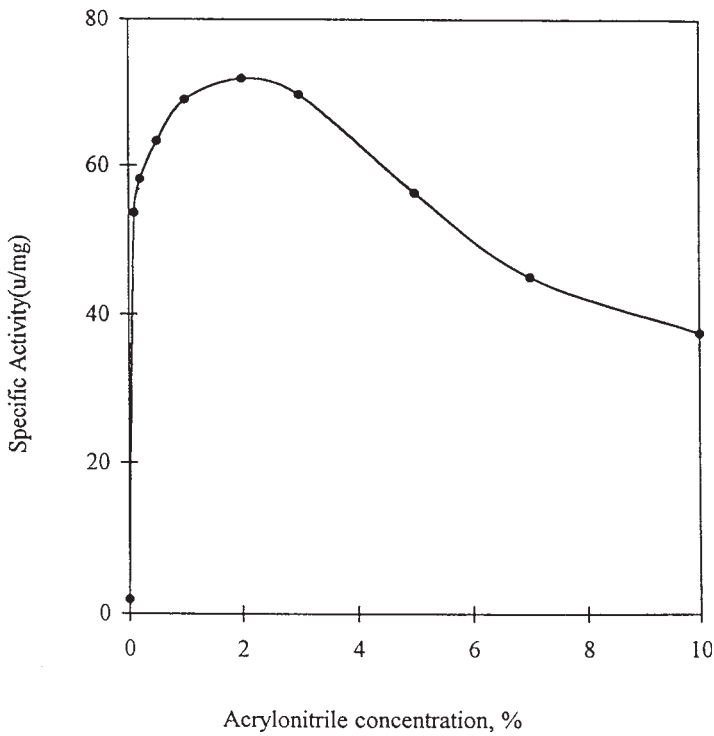

Fig. 1. Nitrile hydratase activity with varied acrylonitrile concentration. Activity was measured at 50°C in 0.05 *M* phosphate buffer, pH 7.5, with the selected concentration of acrylonitrile using suspensions of whole BR449 cells as described in Materials and Methods.

The hydratase of BR449 proved to be quite resistant to acrylonitrile substrate inhibition, with only 47% inhibition in the presence of 10% acrylonitrile during assay using intact cells at 50°C (Fig. 1). BR449 as well as the other isolates produced nitrile hydratase constitutively, allowing use of rich OP medium rather than nitrile-containing DP medium for production of cells used in bioconversion experiments.

Table 2
Effect of Metal Ions on Nitrile Hydratase Activity

Metal ion	Total cells (mg/L, dry weight)	Specific activity (U/mg cell)	
		Nitrile hydratase	Amidase
None	90	3.2	1.8
Co^{2+}	225	71.5	9.7
Cu^{2+}	90	1.9	1.5
Fe^{2+}	490	4.3	2.3
Mn^{2+}	65	2.2	2.1
Ni^{2+}	80	6.8	3.4

Identification of BR449

The 1.4 kb PCR product encoding the BR449 16S ribosomal gene was sequenced and analyzed using BLAST. The sequence proved approximately 97% identical to that of *Bacillus* sp 13 DSM 2349 (data not shown). Although little taxonomic information is available for this member of the moderate thermophile group, it is differentiated from the other members by lack of extracellular amylase activity *(10)*. Extracellular amylase was not detected with isolate BR449, supporting the conclusion of similarity based on DNA sequence comparisons.

Effect of Additions on Nitrile Hydratase Activity

Divalent metal ions were added during growth of isolate BR449 on OP medium to provide indications of metals stimulatory to nitrile hydratase production. As shown in Table 2, addition of divalent cobalt increased the specific activity of nitrile hydratase expressed by the cells 22-fold. This finding suggests that the BR449 nitrile hydratase may be a cobalt-containing enzyme family, which includes the *Rhodococccus rhodochrous* J1 and *Pseudomonas putida* NRRL-18668 nitrile hydratases *(4,11)*.

Temperature and pH Optimum of BR449

BR449 showed a broad pH optimum for activity, with a maximum near pH 7.5 (Fig. 2). A very broad temperature optimum was also observed, with optimum at 55°C (Fig. 3). In separate enzyme-stability studies, the BR449 nitrile hydratase proved stable during 2-h incubations in buffer at temperatures to 60°C, with inactivation ensuing above this temperature (Fig. 4).

Acrylamide Production

In preliminary experiments to determine rates of acrylamide production with temperature, 5 mg (dry weight) BR449 cells were incubated with shaking in 10 mL 2% acrylonitrile, 0.05 *M* phosphate buffer, pH 7.5. As seen in Fig. 5, initial reaction rates at higher temperatures were initially rapid, but ceased after 1 h or less. This decrease in reaction rate proved to be owing

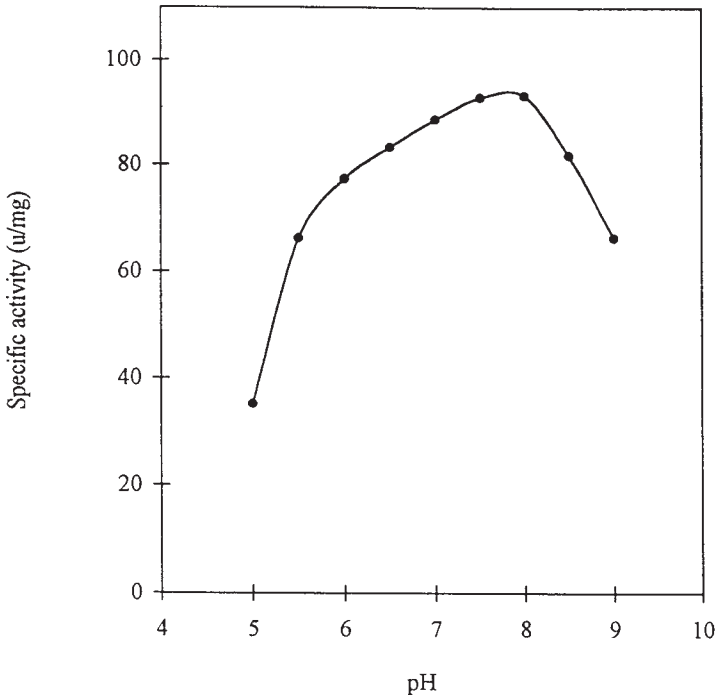

Fig. 2. pH dependence of BR439 nitrile hydratase assayed at 50°C. Activity was measured using whole BR449 cells suspended in 0.05 *M* phosphate buffer at the pH indicated and 0.5 *M* acrylonitrile as described in Materials and Methods.

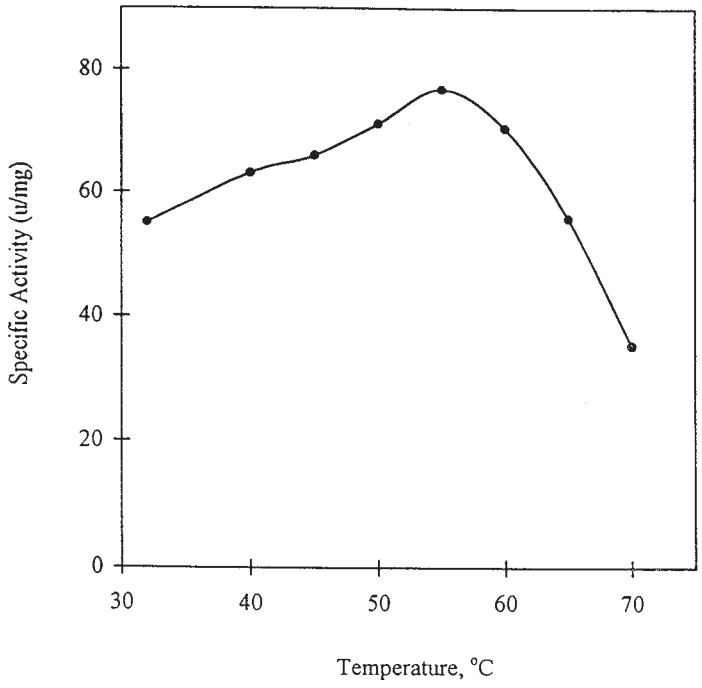

Fig. 3. Nitrile hydratase activity at varied temperatures. Activity was measured over a 10-min period at the selected temperature using whole cells of BR449 suspended in 0.05 *M* phosphate buffer, pH 7.5 containing 0.5 *M* acrylonitrile.

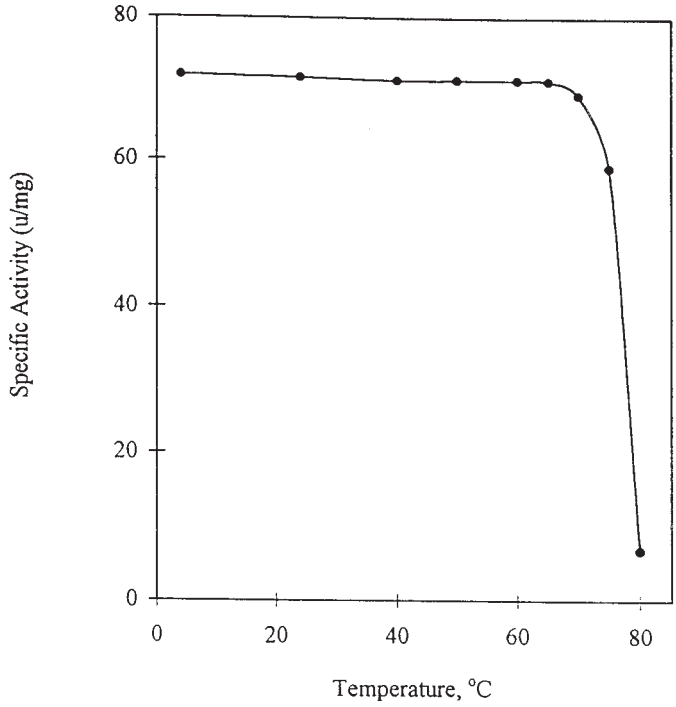

Fig. 4. Nitrile hydratase stability. Nitrile hydratase activity was measured in standard assay at 50°C after 2 h incubation in 0.05 *M* phosphate buffer, pH 7.5, at the temperature indicated.

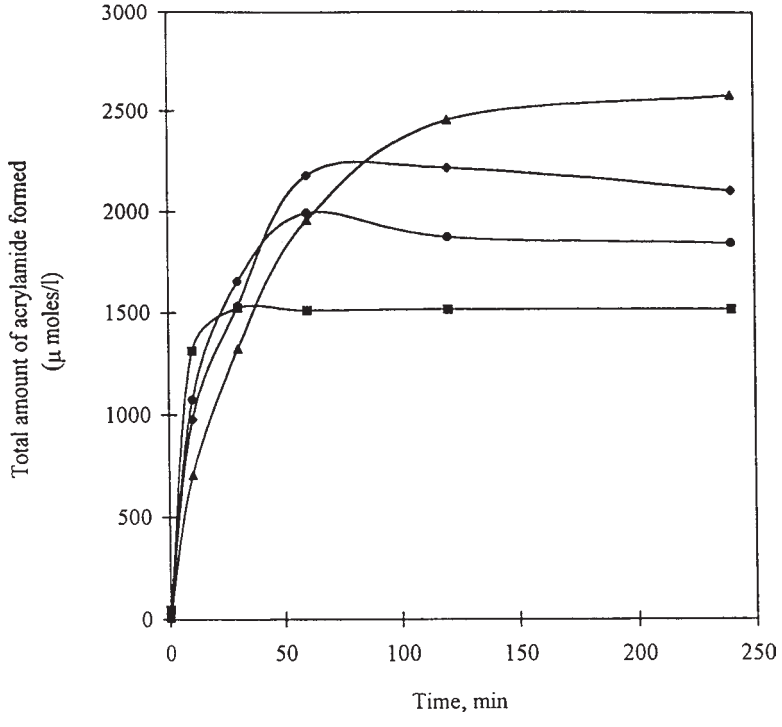

Fig. 5. Acrylamide production at varied temperatures using whole BR439 cells in 0.05 *M* phosphate buffer, pH 7.5, containing 2% acrylonitrile. ■, 22°C; ●, 30°C; ◆, 40°C; ▲, 50°C.

to catalyst inactivation, as dilution with fresh substrate was without effect (data not shown). Although the initial reaction was slower, lowering the reaction temperature to 22°C resulted in prolonged catalyst lifetime and increased product formation.

Discussion

We have determined that aerobic thermophiles that are able to grow in acrylonitrile concentrations of 1% can readily be isolated. *Bacillus* sp. BR449 proved of particular interest because of its high nitrile hydratase activity and tolerance to acrylonitrile. The large increase in nitrile hydratase specific activity in this isolate as a result of cobalt addition to the growth medium suggests that the BR449 nitrile hydratase may be of the cobalt-containing family. Although the BR449 hydratase resists inhibition by concentrated acrylonitrile in shorter term whole-cell assay at 50°C, longer term incubations in 2% acrylonitrile resulted in highly temperature-dependent inactivation, even at temperatures where the enzyme proved stable in buffer. Because the alkylation of the nucleophilic protein residues histidine and cysteine by vinyl compounds including acrylonitrile is well-established *(12)*, we interpret the inactivation as owing to acrylonitrile alkylation of nucleophilic enzyme residues important for conformational stability and/or catalytic activity, and at enzyme locations vulnerable to attack such as the active site. Thus, although the nitrile hydratase of *Bacillus* sp. BR449 exhibits a number of attractive scientific and biotechnological attributes, it is hoped that future research directed toward identification and site-directed replacement of the alkylation-sensitive residues will provide an even more effective catalyst for acrylonitrile industrial bioconversions at elevated temperatures.

Acknowledgment

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