Cyanidase from Bacterial Sources and its Potential for the Construction of Biosensors

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Abstract

Because of their content of cyanogenic glycosides, many medicinal and food plants are toxic for man. If plant material containing cyanogenic glycosides gets disintegrated, cyanide is liberated by the action of different enzymes. Especially in developing countries, chronic poisoning by cyanogenic plants is a serious problem. Since probably more than 2500 plant species and also some insects contain cyanogenic glycosides, a rapid and precise method for the determination of these compounds should be developed. A biosensoric system based on an ammonia electrode and the enzyme cyanidase [EC 3.5.5.1] seems to be an effective analytical method for this class of substances and a promising alternative to an ion-selective cyanide electrode. The key-step in the development of such a sensor is the selection of a suitable cyanidase, which has been previously reported for bacteria. This biosensor should be used for screening purposes as well as for the quality control of cyanogenic medicinal and food plants. For this reason, we have examined strains of the bacteria *Rhodococcus rhodochrous*, *Alcaligenes xylosoxidans*, and *Acinetobacter spec.*. Strains were fed with increasing concentrations of potassium cyanide in order to induce cyanidase activity. After three cycles of selection, *Alcaligenes xylosoxidans* exhibited sufficient growth at cyanide concentrations up to 2×10^{-3} M. However, *Rhodococcus rhodochrous* showed excellent performance even at concentrations as high as 1×10^{-2} M cyanide. In addition, the latter bacterium is able to digest isovaleronitrile. No significant inhibition of growth was observed at concentrations up to 2×10^{-2} M isovaleronitrile. Because of cyanidase activity, *Acinetobacter spec.* was capable to utilize cyanide as nitrogen source. First measurements with immobilized cyanidase in a flow-through apparatus based on an ammonia electrode gave a detection limit at 0.2 mg/L cyanide. The linear range of the calibration curve was between 0.6 mg/L and 30 mg/L cyanide.

Introduction

Because of their content of cyanogenic glycosides, many medicinal and food plants are toxic for man [1, 2]. Cyanogenesis is known to occur in the plant genera *Prunus*, *Sambucus*, *Linum*, *Carica*, *Phaseolus* and *Manihot*. Mainly plants belonging to the genera *Phaseolus* and *Manihot* are widely used as food. Typical cyanogenic glycosides like amygdalin, linamarin, and lotaustralin, which are shown in Figure 1. If plant material containing cyanogenic glycosides gets disintegrated, cyanide is
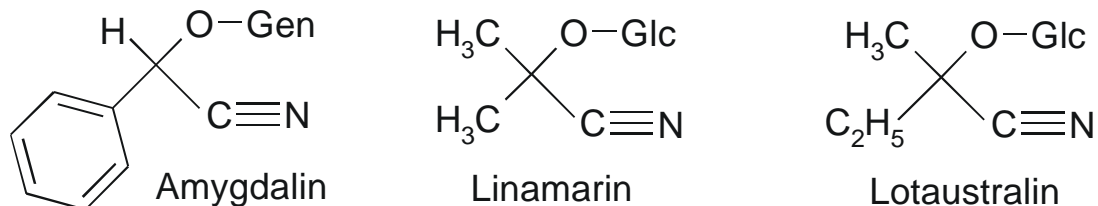

Fig. 1 *Typical cyanogenic glycosides which occur in nature.*

liberated by the action of different enzymes. Cleavage of the glycoside residue by â–glycosidases is the most prominent step in this procedure. Especially in developing countries, chronic poisoning by cyanogenic plants after the consumption of "Manihot" is a serious problem. These poisonings are either cause by insufficient heating of food or by manihot with high levels of cyanogenic glycosides.

Since probably more than 2500 plant species and also some insects contain cyanogenic glycosides, a rapid and precise method for the determination of these compounds should be developed. A biosensoric system based on an ammonia electrode and the enzyme cyanidase [EC 3.5.5.1] seems to be an effective analytical method for this class of substances and a promising alternative to an ion-selective cyanide electrode [3].

The key-step in the development of such a sensor is the selection of a suitable cyanidase, which has been previously reported for bacteria [4, 5]. However, this enzyme is not commercially available at time. Therefore, a number of bacteria species should be screened for cyanidase activity in the present study. Investigations will be focused on induction of enzymatic activity.

Fig. 2 *Principle of function of the proposed biosensor for the detection of cyanogenic glycosides.*

The resulting sensor will be similar to the biosensoric system for alliin detection [6].

Cyanide should be analyzed in extracts obtained from cyanogenic plants. In effect, determination of cyanogenic glycosides includes two enzymatic steps (Fig. 2).
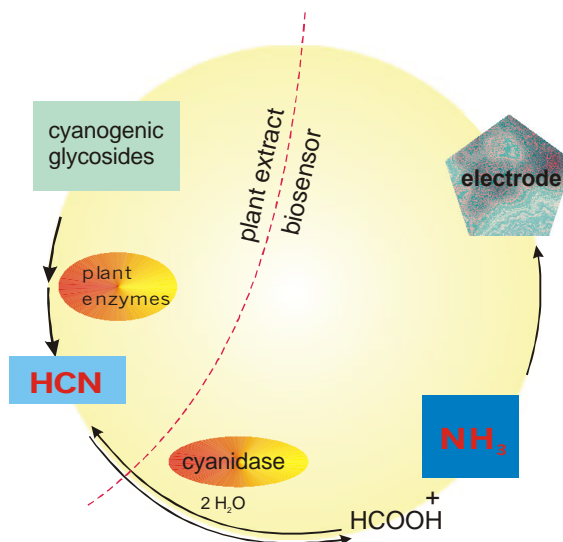
Materials and Methods

Reagents. Unless otherwise stated, chemicals were purchased either from Merck (Darmstadt, Germany) or Fluka (Deisenhofen, Germany) and were purified by standard procedures. Water for preparing buffers and standards was purified with a Millipore-Q-Reagent-system (Millipore, Bedford, MA, USA). Ammonium-free chemicals, like NaOH and phosphate salts, were purchased from Merck (Darmstadt, Germany) in suprapur®-quality.

Cultivation of bacteria. Strains of *Rhodococcus rhodochrous*, *Alcaligenes xylosoxidans*, and *Acinetobacter spec.* (ATCC 333) were obtained from the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Bacteria were cultivated in Erlenmeyer flasks (250 mL) containing liquid culture media (100 mL). Standard culture media contained polypeptone (5 g), malt extract (3 g), and yeast extract (3 g) solved in 1000 mL water (pH
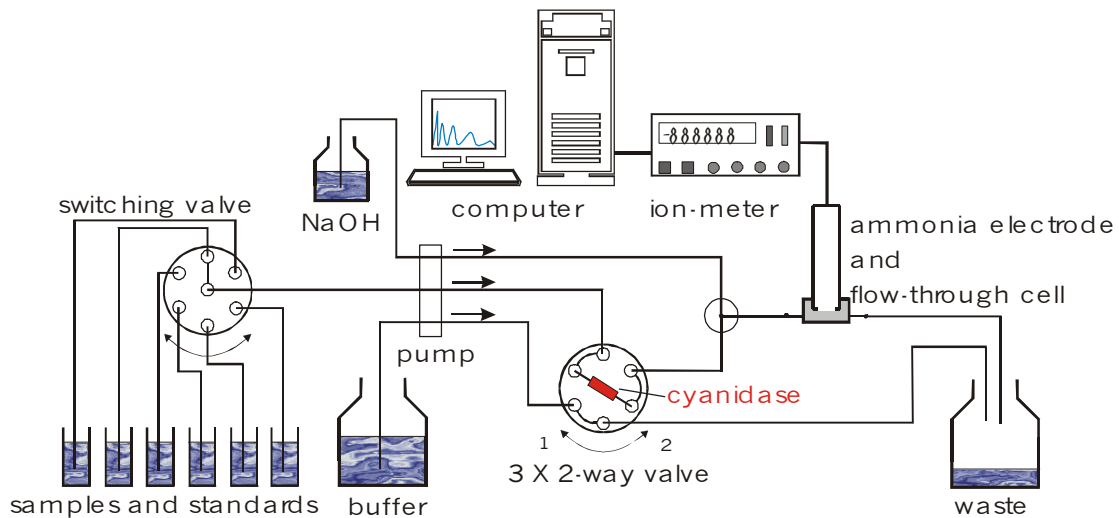

Fig. 3 Flow-through apparatus used for the determination of cyanide. Cyanidase was immobilized inside a cartridge.

7.5). *Acinetobacter spec.* was cultivated on peptone (5 g) and meat extract (3 g) solved in 1000 mL water (pH 7.0). The medium used for experiments with reduced nitrogen source (M9) contained Na_2HPO_4 x $5H_2O$ (13 g), KH_2PO_4 (3 g), NaCl (0.5 g), NH_4Cl (1 g), and glucose (5 g) solved in 970 mL water. In order to avoid evaporation of hydrocyanic acid, bacteria were cultivated on a shaker at room temperature (22 °C). A sample of 5 mL was taken at the end of the exponential growth phase of bacteria and was transferred into a new flask. Bacteria growth was monitored by determination of the OD 600.

Flow-through apparatus. A flow-through apparatus was used for enzyme-based analysis (Figure 3). The "analyzer" consisted of a peristaltic pump (type MC-MS/CA4/8, Ismatec, Glattbrugg, Switzerland), a 6-port switching valve and a 3 x 2-way valve (Knauer, Berlin, Germany), an Orion (Beverly, Mass., USA) 95-12 ammonia electrode fitted to a flow-through cell with a cell volume of 2 µL. A cartridge containing 284 mg immobilized cyanidase (Novo Nordisk Sp 379) was integrated into the flow line.

The electrode was connected to a pMX 3000 ionmeter (WTW, Weinheim, Germany). The analyzer was fully computer controlled by a PC equipped with EUROCHROM 2000 TM software (Knauer). Samples for analysis were diluted in phosphate buffer (pH 8.0; 0.02 M containing 0.17 M NaCl) and delivered with a flow rate of 0.5 mL/min. The buffer stream was basified with a NaOH solution (0.5 M, flow rate 0,025 mL/min). Freshly prepared cyanide standards in a concentration between 5 x 10^{-6} M and 1 x 10^{-3} M were used for the determination of substrate specificity. The analyzer is described in detail in references [7, a].

Results and Discussion

In order to find suitable bacteria for the isolation of cyanidase, three different strains belonging to the genera *Acinetobacter*, *Rhodococcus*, and *Alcaligenes* were selected for experiments. The latter bacterium was chosen, because the Novo Nordisk Company recently found cyanidase in *Alcaligenes*. However, this enzyme is not commercially available and our research group therefore performed independent induction experiments. The strategies for induction of enzymatic activity were deduced from recently published methods [4, 5].

Firstly, *Alcaligenes xylosoxidans* was cultivated in media containing 2 x 10^{-3} M cyanide. Most resistant bacteria were selected and again exposed to cyanide. After 3 cycles of selection, *Alcanigenes xylosoxidans* exhibited sufficient growth at cyanide concentrations up to 2 x 10^{-3} M (Fig. 4). However, even after induction of cyanidase activity over three cycles, no further tolerance towards higher cyanide concentration could be detected. It must be assumed, that *Alcaligenes xylosoxidans* is
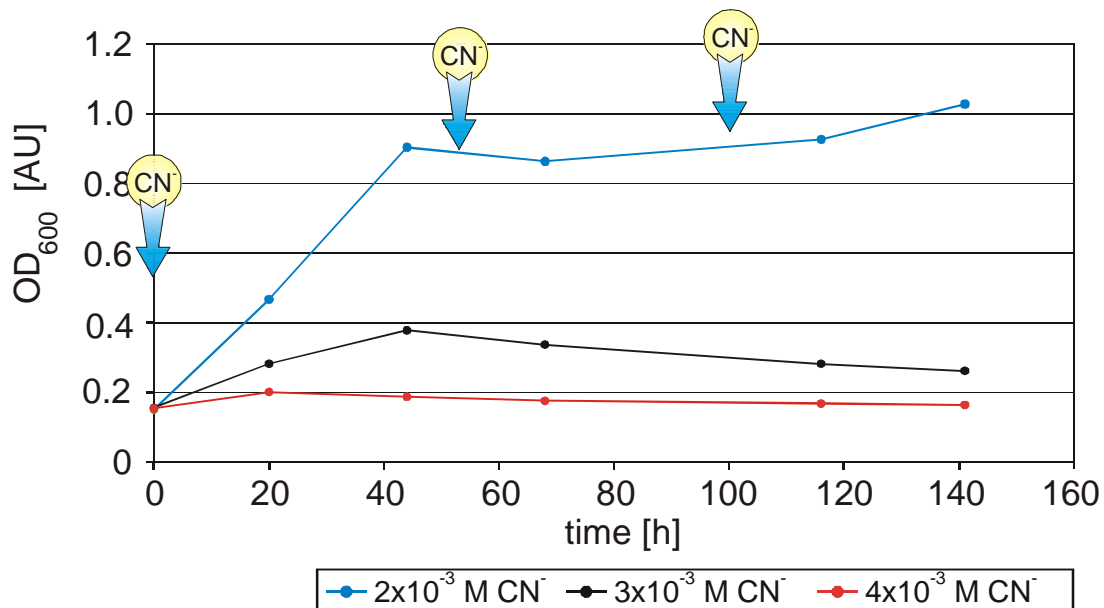

Fig. 4   Growth curves of Alcaligenes xylosoxidans. The addition of cyanide is marked by arrows.

capable to digest cyanide enzymatically. However, this ability is restricted to lower cyanide concentrations.

In contrast to the first set of experiments, *Rhodococcus rhodochrous* was fed with isovaleronitrile for the first cycles of growth [4]. It has been reported, that this bacteria is also capable to utilize nitriles, which were hydrolyzed by the enzyme nitrilase. Nitrilases and cyanidases seem to be closely related enzymes. When growth of *Rhodococcus rhodochrous* was sufficient, selected bacteria were fed with cyanide at increasing concentrations ranging from 1×10^{-3} to 1×10^{-2} M. Every 48 h cyanide was added. Strains were cultivated in nutrient broth medium at pH 7.5. Additionally, bacteria were fed with isovaleronitrile.

Rhodococcus rhodochrous showed an excellent performance even at concentrations as high as 1×10^{-2} M of cyanide (260 mg/l). In addition, the bacterium is able to digest isovaleronitrile. No significant inhibition of growth was observed at concentrations up to 2×10^{-2} M isovaleronitrile (Fig. 5). Consequently, *Rhodococcus rhodochrous* is capable to digest both cyanide and nitriles in relatively high concentrations. Results are more promising than those obtained for *Alcaligenes xylosoxidans*
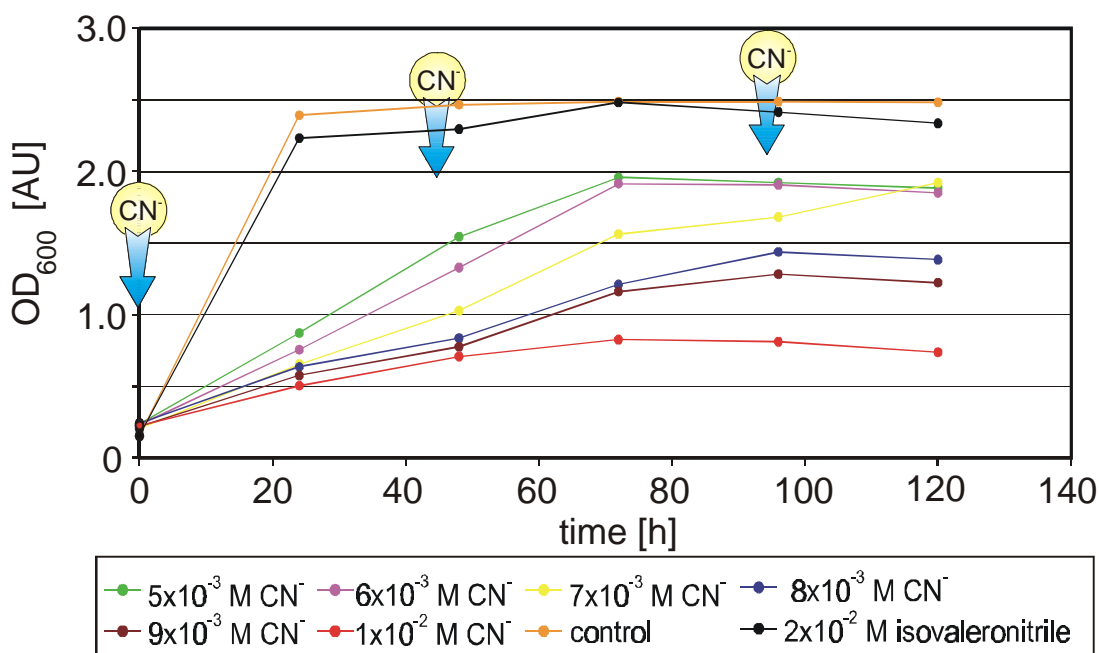

Fig. 5   Growth curves of Rodococcus rhodochrous. The addition of cyanide is marked by arrows.

(Fig. 4). *Rhodococcus rhodochrous* can be considered as source for the isolation of cyanidase. However, separation of nitrilase and cyanidase might be crucial. Both enzymes seem to be active in *Rhodococcus rhodochrous*. Whole cells of *Rhodococcus rhodochrous* might be immobilized and combined with an ammonia electrode to give the final biosensor (Fig. 2). Alternatively, the enzyme might be isolated followed by immobilization. The resulting sensor will be similar to the biosensoric system for alliin detection (6, 7, a).

An *Acinetobacter* strain (*Acinetobacter* spec.) was investigated in a somewhat different manner. In a first set of experiments, bacteria were exposed to cyanide in a concentration of 2×10^{-3} M. Bacteria showing good performance were selected and transferred to the M9 medium (reduced nitrogen source). Then, *Acinetobacter* was periodically fed with 0.2 mmol cyanide (Fig. 6). Bacteria of the

control experiment with no cyanide showed significant growth between 10 and 50 hours of cultivation. Then, growth nearly stopped. Interestingly, bacteria exposed to cyanide did not significantly grow up to 50 hours. After this increased lack-phase, growth started and performance after 70 hours was better than that of bacteria of the control experiment.
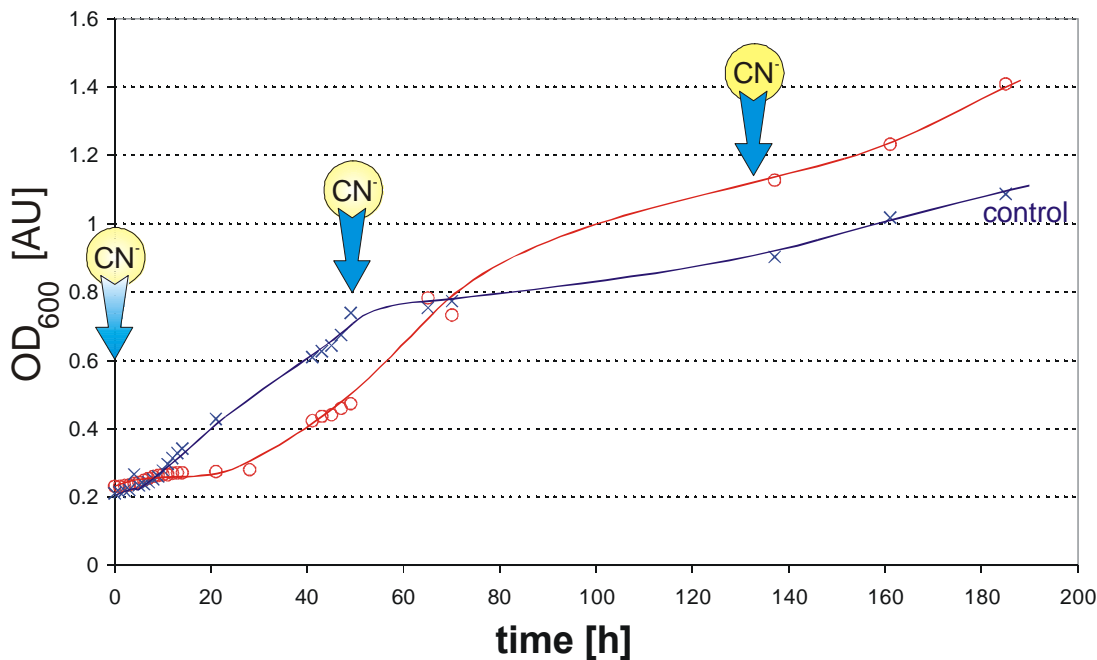

Fig. 6   Growth curves of Acinetobacter spec. The addition of cyanide (2 mmol) is marked by arrows.

This experiment demonstrates that cyanidase activity can be induced in the investigated *Acinetobacter* strain. But this process needs about two days. During this period, metabolism of *Acinetobacter* will be adapted the cyanide. Under the used conditions, bacteria are forced to utilize cyanide. *Acinetobacter* seems to be a promising source for the production of cyanidase. Further experiments are planed in order to utilize cyanidase of *Acinetobacter* for the construction of a cyanide biosensor. As described above, immobilization either of whole cells or of the isolated enzyme might be possible.

Finally, a sample of cyanidase obtained from Novo Nordisk was tested in the flow-through apparatus as shown in Figure 3. This enzyme isolated from *Alcaligenes xylosoxidans* was already immobilized. However, no information was given by the producer about the immobilization procedure. This cyanidase is not commercially available and was used for a "proof a principle".

Cyanide degradation was monitored between 0.1 mg/L and 30 mg/L. The detection limit was found to be at 0.2 mg/L (Fig. 7). The linear range of the calibration curve was between 0.6 mg/L and 30 mg/L. Experiments were performed over a duration of four weeks. No loss in enzyme activity was observed over this period.

In conclusion, it could be demonstrated that a biosensoric determination of cyanide seems to be possible. The sensitivity of the flow-through method is comparable to conventional methods, *e.g.*, as described in DIN 38405. From experiments with the Orion® ammonia-gas electrode described in ref. [7, a] it can be assumed that sensitivity of the system can be increased by at least one order of magnitude. Additionally, conventional methods include a distillation step. If cyanides are not bounded by metals, the distillation step is unnecessary for the biosensoric method. This is also the case for cyanogenic glycosides so that this class of compounds can be determined by a biosensoric method without complex sample preparation.

Cyanogenic glycosides can be expected in concentrations between 0.1 % (flax seeds) and 8 % (almond seeds). Percentages are related to the fresh weight of seeds. Taking into account the detection limit mentioned above, between 3 mg and 250 mg of a fresh sample, extracted in 100 mL buffer, are necessary to
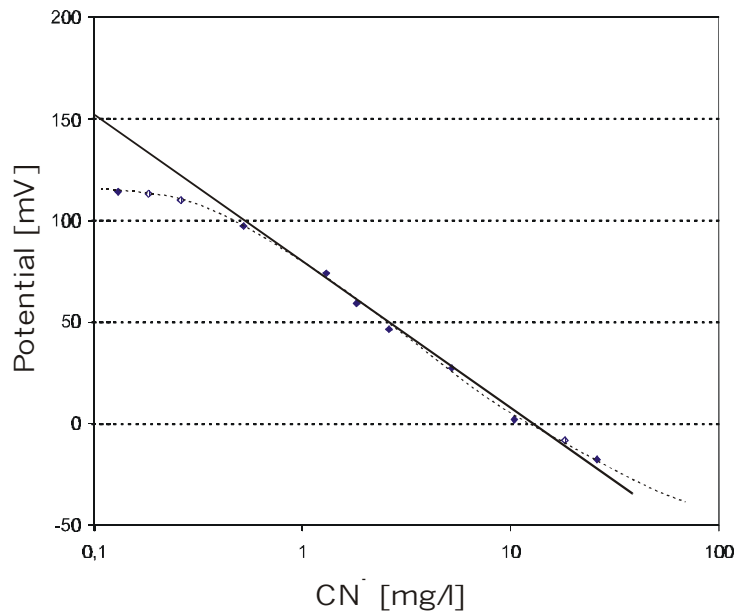

Abb. 7 Calibration curve obtained with cyanidase from Alcaligenes xylosoxidans (Novo Nordisk SP 379). Measurements were performed with the apparatus shown in Figure 3.

perform analysis by the described method. This sensitivity is high enough even to analyze single seeds. Thus, the method seems to be suitable for the aspired use. In the next step of investigation, real samples will be analyzed. Additionally, the cyanidase will be immobilized directly at the surface of an ammonia detecting device (*e.g.*, ammonia gas electrode).

Acknowledgements

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