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Degradation of Cyanide in Cassava (*Manihot esculenta)* Plant Leaves by *Pseudomonas* sp.

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Cyanogenic toxic compounds are found in the environment because of pollution, natural synthesis by some plants, microorganisms and by some industrial effluents. The biodegradation of these cyanide containing compounds prevailing in the environment was studied by using the isolated strains of *Pseudomonas fluorescens and Pseudomonas aeruginosa*. Linamarin, one of the cyanogenic glycoside present in the leaves and roots of Cassava crop was extracted, mixed with the suitable medium and incubated with the test organisms for degradation. Results revealed that the *Pseudomonas fluorescens* alone has the ability to degrade the cyanogenic compound in cassava leaves. The degradation was confirmed by the color change of alkaline picrate paper and the release of ammonia. Also the experiment was carried out at different pH and temperature to determine the optimal conditions for the efficient biodegradation.

Key Words: *Pseudomonas fluorescens, Pseudomonas aeruginosa,* Cassava crop Cyanogen, Linamarin.

Cyanides are synthesized by a range of organisms including higher plants, fungi and bacteria. There are about 2000 plant species that produce sufficient amounts of cyanogenic compounds which functions as the translocatable forms of reduced nitrogen and chemical defense molecules against pests and diseases on plants.

In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and serve the plant as defense against herbivores[1]. There are different forms of cyanogens in higher plants including amygdalins occurring in the family Rosaceae, Linamarin occurring in the Cassava plant, Dhurrin occurring in Sorghum and Linmarin occurring in Limabeans [2].

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Cyanogenic glycosides, on hydrolysis yield a ketone or aldehyde, a sugar and the higher toxic cyanide ion[3]. Cassava (*Manihot esculenta*), the third most important food source in the tropics produces two cyanogenic glycosides, linamarin and a small amount of lataustralin (Methyl linamarin). These cyanogenic glycosides are hydrolysed in the presence of the enzyme linamerase (β-glycosidase) to a cyanohydrin, which breakdown further to produce hydrogen cyanide and acetone[4]. Linamarin in Cassava is synthesized in leaves and petioles, glycosylated within cells to linustatin, transported via apoplast to roots and stored in root cells[5].

Many microorganisms (bacteria and fungi) have developed resistance and possess the ability to degrade toxic cyanide compounds by number of mechanisms[6]. Having this background, the present investigation is focused on the identification of cyanide degrading organisms from various sources and to identify their spectrum of degradation.

MATERIALS AND METHODS

Sample collection

The samples such as garden soil, cassava peel, cassava plant soil, were collected from Punalur, Kollam District, Kerala State, and transported without any contamination to the lab.

Isolation of test organisms

The samples collected were plated on to sterile selective media of Pseudomonas isolation agar and incubated at 37°C for 24- 48 hours. Two different species of *Pseudomonas* were identified and their species level conformation as *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* was done by the results obtained in the standard biochemical tests as per the Bergey's Manual of Determinative Bacteriology. Further, these two organisms were used for the biodegradation of cyanogenic compound extracted from cassava leaves.

Separation of Linamarin from Cassava leaves

Young cassava leaves were collected for about 5 gms and ground in a glass pestle and mortar with 0.1 M HCl and made into a pasty solution by adding 5ml of 0.1 M HCl again. This solution was passed through a muslin cloth, which was then squeezed. The pink colored cloudy solution thus obtained was centrifuged and the clear supernatant liquid formed was removed by using a Pasteur pipette. This solution contains linamerase (inactivated in 0.1 M HCl) and linamarin was stored frozen at -20°C. The degradation of linamarin was confirmed by alkaline picrate paper test and by the ammonia released.

Table 1. Cyanide degradation pattern of the test organisms

Test Organisms	HCN Production	Color change of Picrate paper	Ammonia Released	Reaction with Nessler's reagent	Cyanide degradation
Pseudomonas fluorescens	+	From yellow to brown	+	From pale yellow to dark yellow	Positive
Pseudomonas aeruginosa	–	No color change	–	No color change	Negative

Table 2. Effect of pH and temperature on degradation of cyanide by *Pseudomonas fluorescens*

pH	OD Value (550nm)	Temperature	OD Value (550nm)
5.0	0.231	30°C	0.010
6.0	0.761	35°C	0.035
7.0	0.908	40°C	0.079
8.0	1.028	45°C	0.121
9.0	0.991	50°C	0.005

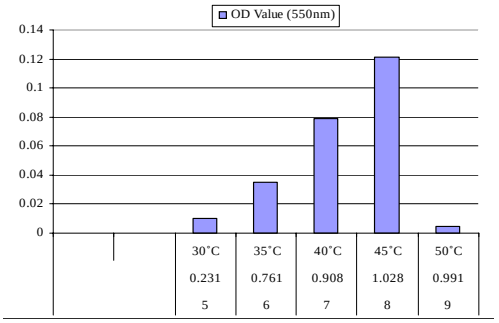

Fig 1. Effect of tempreture and pH

Preparation of alkaline picrate paper

Alkaline picrate papers were made by dipping the strips of Whatman No 1 filter paper (1.0- 2.5 cm) in a solution of alkaline picrate prepared by mixing equal volumes of saturated picric acid and 5% (w/v) Na_2Co_3. It was allowed to dry. These prepared picrate papers were used to determine the microbial degradation of linamarin (cyanide).

Degradation of Cyanogenic Glycosides (Linamarin)

Modified agar medium was prepared,

sterilized and the cyanogenic glycoside (Linamarin) was incorporated into the medium before plating. The isolated test bacterial strains were streaked on the agar plates. The sterilized picrate paper was placed above the streaked area on the plate and was incubated at 37°C for 4 days. After incubation, the color change of picrate paper (yellow to brown) was observed to determine the cyanide degradation.

Determination of Ammonia released

The isolated cyanide-utilizing bacteria, was inoculated into 10ml of sterile peptone water containing Linamarin and incubated for 48- 72 hours at 37°C. After incubation, 0.5mlof Nessler's reagent was added into tubes and the color change was observed (from pale yellow to dark yellow).

Effect of pH and temperature

Temperature and pH are the main factors that affect the biodegradation of cyanogenic glycosides. To determine the effective condition for the maximum degradation, the organism was tested at different conditions of pH and temperature.

The organism was inoculated into 100ml of M9 medium containing various pH (5, 6, 7, 8 and 9). The linamarin was incorporated into these broth medium and incubated at 37°C for 24 hours. After incubation, growth in the culture suspension was measured by spectrophotometer at 550nm. To study the effect of temperature, the same procedure was followed at 30, 35, 40, 45 and 50°C and incubated at the constant pH of 7.5.

RESULTS

Degradation of Cyanogenic glycoside

Isolated *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were tested for their degradation ability and it was detected by the breakdown of linamarin resulted in the volatilization of HCN, which cause discoloration of alkaline picrate paper. Between two *Pseudomonas species*, *Pseudomonas fluorescens* alone has changed the color of picrate paper from yellow to brown and it was found as an efficient cyanide-degrading organism Table 1.

Determination of Ammonia production

As a result of cyanide degradation, ammonia is released as the end product. To confirm the cyanide degradation by the test organisms, ammonia production in the reaction mixture was identified by adding Nessler's reagent. *Pseudomonas fluorescens* degraded the cyanide compound and released ammonia, which was identified by the formation of distinct yellow color with Nessler's reagent. But *Pseudomonas aeruginosa* showed negative result for ammonia production Table 1.

Optimization of Biodegradation

Effect of pH

To find the optimum pH for the maximum degradation, the medium was maintained at different pH (5, 6, 7, 8 and 9).The maximum level of cyanide degradation was observed at pH 8 (1.028 OD at 550nm) by *Pseudomonas fluorescens* (Table 2, Fig1**)**.

Effect of Temperature

Optimum temperature for degradation was also measured by providing different temperature ranges (30, 35. 40, 45 and 50°C). Cyanide degradation by *Pseudomonas fluorescens* was gradually increased up to 45°C and the maximum degradation (0.121 OD at 550nm) was noted at 45°C (Table 2, Fig 1).

DISCUSSION

In the present study, *Pseudomonas fluorescens* was found to effectively degrade the cyanogenic glycoside, linamarin of cassava. Similarly, *Pseudomonas fluorescens* isolated from the soil was found to have the ability to degrade simple cyanide and metal cyanide mainly cuprocyanide, ferro- ferric cyanide and thiocyanates formed from the cyanidation process of gold extraction[7].

Immobilized cells of *Pseudomonas putida* was found to utilize cyanide as the sole source of carbon and nitrogen and degraded cyanides, cyanates and thiocyanates to ammonia and carbon dioxide[8]. In this work *Pseudomonas fluorescens* was also found to degrade cyanide compound and released ammonia as the end product.

Normally *Pseudomonas fluorescens* catalyzed the degradation of cyanide into products that induce $Co_{2,}$ formamide, formic acid formation by the activity of enzymes cyanide oxygenase, cyanide nitrilase (dihydratase) and hydratase[9].

It was reported that the picrate paper kits were used to determine the cyanide degradation[10]. With this reference in this study, the color change of the picrate paper from yellow to brown was used as the indicator to determine the occurrence of cyanide degradation. This color change occurred due to the formation of hydrogen cyanide from linamarin by the activity of linamerase enzyme. To confirm the degradation in the present study, release of ammonia as the end product was detected by using Nessler's reagent, which on reaction with the incubated medium of linamarin and the test organism showed the color change from pale yellow to dark yellow.

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