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Seeds of rosary pea, *Abrus precatorius*: A novel source of hydroxynitrile lyase

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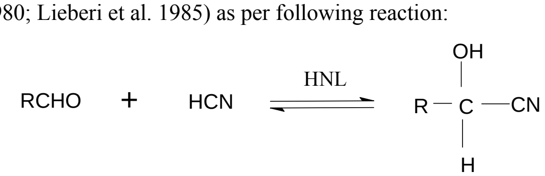
Abstract

Seeds of *Abrus precatorius* (rosary pea) were identified as a new and potential source of hydroxynitrile lyase. Hydroxynitrile lyase (HNL) from the seeds of this plant was purified up to 9 fold with specific activity of 577 Umg^{-1} protein by using ion exchange and gel filtration chromatographic techniques. The purified enzyme was a heteromer with estimated native molecular mass 205 kDa and its subunits showed two bands of molecular mass of 42.0 and 36.5 kDa in SDS-PAGE, respectively. The enzyme exhibited maximum activity at 30ºC with 0.1 M sodium citrate buffer (pH 5). It has K_M of 13 mM and V_{max} of 625 Umg^{-1} of protein with mandelonitrile as a substrate. In 120 mL of reaction mixture containing 25 ml of benzaldehyde (substrate) and 6.75 mg of purified enzyme produced 17.5 g of mandelonitrile.

Key words: *Abrus precatorius*, Cyanohydrins, Hydroxynitrile lyase, Mandelonitrile

Introduction

Hydroxynitrile lyases (HNL, EC 4.1.2.10, EC 4.1.2.11, EC 4.1.2.37, EC 4.1.2.39) are the versatile group of enzymes that catalyse enantioselective synthesis and cleavage of cyanohydrins (Conn 1980; Lieberi et al. 1985) as per following reaction:



Aldehyde Hydrogen cyanide Cyanohydrin

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The catalytic ability of HNLs to form carbon-carbon bond has made these enzymes as important biocatalysts for the synthesis of a range of cyanohydrins in organic chemistry (Pouchlauer 1998). Cyanohydrins or their derivatives are finding wide applications in pharmaceuticals, agrochemicals and cosmetics since these can be readily converted into a myriad of α-hydroxy carboxylic acids, α-hydroxy ketones and β-amino acids (Gregory 1999).

Plants are the major sources of HNLs as these enzymes liberate HCN from naturally occurring cyanohydrins in plant systems and this provides general protection against the bacterial and fungal infections (Albers and Hamann, 1934) and it is also used as a nitrogen source by some plants (Goischmidt et al. 1963). HNLs have been reported, purified and characterized from almond (*Prunus amygdalus*), flax (*Linum usitatissimum*), apple (*Pyrus malus*), apricot (*Prunus armeniaca*), cherry (*Prunus serotina*), peach (*Prunus persica*), capulin (*Prunus capuli*) and rubber tree (*Hevea brasillensis*) etc. (Ingrid et al. 2001; Klaus et al.1997; Tuncel et al. 1995; Asano et al. 2005; Ueatrongchit et al. 2010; Forster and Wajant 1996). In the present investigation some plants of Himachal Pradesh were screened with an objective to find new source of HNL. The purification and characterization of HNL from a new source *i.e.* rosary pea (*Abrus precatorius* (Leguminaceae) was undertaken because of its easy availability in the forests of Himachal Pradesh and processivity of its seeds as compared to seeds of the stone fruits of rosaceaae family and thus it has the potential to emerge as an important source of HNL for the enzymatic synthesis of cyanohydrins.

Materials and Methods

Source of chemicals

Potassium cyanide (KCN), ethyl acetate, benzaldehyde used in the present study were purchased from SD Fine Chemical Ltd., India. Mandelonitrile was purchased from Merck India Ltd. and other chemicals were of analytical grade procured from various commercial sources.

Plant material

A number of plants *Abrus precatorius* (seed), *Carissa carundus* (seed), *Eribotrya japonica* (seed), *Ficus elastica* (leaves), *Pinus girardiana* (seed), *Prunus amygdalus* (seed), *Prunus armeniaca* (seed), *Prunus domestica* (seed), *Prunus serotina* (seed) and *Pyrus malus* (seed) were collected from various places in Himachal Pradesh, India and screened for HNL activity.

Preparation of crude HNL

Crude HNL was prepared following the method described by Han (Han ***et al.*** 2001). The seeds of *Abrus precatorius* were soaked in water for 96 h at room temperature. The presoaked seeds or leaves were ground in mortar- pestle with chilled ethyl acetate and air dried at 4 ºC. The dried powder material was termed as 'meal'. The crude enzyme was prepared by suspending this meal in distilled water (7.4 g/100mL), adjusting its pH to 7.4 with 1N NH_4OH. The suspension was incubated overnight at 4 ºC and then its pH was adjusted at 5.4 with 50 % acetic acid. To suppress proteolysis, protease inhibitor phenylmethanesulphonylfluoride (PMSF; 10 mg/300mL) was added to the enzyme preparation. The protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Assay of hydroxynitrile lyase (HNL) activity

The activity of HNL was measured according to the procedure of Willeman *et al* (2000). The assay mixture (5 mL) contained 0.1 M sodium citrate buffer (pH 5) and 1 mg crude, enzyme protein and 250 µmole of mandelonitrile (benzaldehyde cyanohydrin). It was incubated at 30 ºC for 30 min and the reaction was quenched by adding 5 mL trichloroacetic acid (TCA).The reaction mixture was centrifuged at (10,000 x *g*) in sigma ultracentrifuge and the absorbance of the supernatant was recorded at 280 nm. One unit of HNL activity was defined as the amount of enzyme which catalyses the release of 1 µmole of benzaldehyde in 1 min under the assay conditions. Since (*Abrus precatorius*) rosary pea had maximum HNL activity among various plant materials tested, it was used for further purification and chacterization.

Purification of HNL of rosary pea

All steps of protein purification were performed at 4ºC and 0.1 M sodium citrate buffer (pH 5.0) was used as a buffer throughout the purification process. Centrifugation at (15,000 x *g*) was carried out for 15 min at 4 ºC. The crude enzyme was prepared from 200 g seeds of rosary pea by following the procedure described above.

Ion exchange chromatography

The crude enzyme (21.06 mg protein in 130 mL) was loaded onto DEAE-Sepharose column (9 x 2 cm, bed volume 25 mL). The column was washed with start buffer (0.1 M sodium citrate buffer pH 5). The protein was eluted using elution buffer (0.1 M sodium citrate buffer having 1M NaCl, pH 5) at flow rate of 0.5mL/min and fractions of 4 mL each were collected. The fractions rich in HNL activity were pooled and concentrated by lyophilization.

Gel filtration chromatography

The pooled and lyophilized fractions of ion exchange chromatography were applied to Sephacryl S-100 column (1.6 x 60 cm) using AKTA Prime Liquid Chromatography System. The concentrated protein (2.5 mL) having 6.7 mg of protein was loaded onto pre-equilibrated (sodium citrate buffer pH 5) Sephacryl S-100 column and the protein was eluted using sodium citrate buffer (pH 5) containing 0.15 M NaCl at a flow rate of 0.3 ml/ min and fractions of 4 mL each were collected. Protein fractions rich in HNL activity were pooled and subjected for electrophoresis.

Sodium dodicyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Non-denaturing (Native) –PAGE

The enzyme preparations of various stages of purification were subjected to SDS-PAGE and native PAGE using the procedure of Lacmmli, 1970.

Optimization of reaction conditions for assay of HNL activity

To optimize reaction conditions for assay of HNL activity of rosary pea, enzyme reactions were carried using various buffer systems [phosphate buffer, sodium citrate buffer and sodium acetate buffer of 0.1M ionic strength], buffer pH (3-8) and temperature (15º-60ºC).

Synthesis of mandelonitrile

Mandelonitrile was synthesized by using the protocol of Rhodium (Rhodium, 1991). In a flat bottom flask, 7.26 g of KCN was added to 75 mL of ethyl acetate and kept overnight on magnetic stirrer, then 15 mL of benzaldehyde mixed with 10 mL of ethyl acetate and 15 mL (3894.6 Unit) of rosary pea hydroxynitrile lyase (0.45 mg/mL protein) was added to it and stirring was continued for 24 h at 4˚C. The reaction mixture was filtered and the residues were washed with ethyl acetate. The filtrate was dried and concentrated in vacuum. The mandelonitrile was quantified using HPLC system equipped with Inertsil ODS-2 (4.6 x 150 mm) column, having mobile phase acetonitrile in water (65 % v/v) at the flow rate of 1.0 mL/min.

Result and Discussion

Screening of some plants material for HNL activity

In the present studies, ten different plant materials were screened for hydroxynitrile lyase activity (Table 1) and out of these, the seeds of rosary pea (*Abrus precatorius*) exhibited maximum HNL activity (64 units/ mg of protein).

Table 1: HNL activity in some plants of Himachal Pradesh, India

Name of Plant	Plant part used	Specific activity (units/mg)
Abrus precatorius	Seed	64
Carissa carundus	Seed	50
Eribotrya japonica	Seed	36
Ficus elastica	Leaves	35
Pinus gerardiana	Seed	32
Prunus amygdalus	Seed	59
Prunus armeniaca	Seed	16
Prunus domestica	Seed	35
Prunus serotina	Seed	10
Pyrus malus	Seed	22

Purification of HNL of rosary pea

The purification of HNL of rosary pea (*Abrus precatorius*) was carried out by using ion exchange and gel filtration chromatographies. The ion exchange chromatography was performed using DEAE Sepharose column. The fractions (6 to 10) showing high HNL activity were pooled together and were concentrated to 2.5 mL by lyophilization (Fig. 1). This step resulted in 2.3 fold purification with a yield of 64 % of enzyme (Table 2). The lyophilized sample was loaded on Sephacryl S-100 column

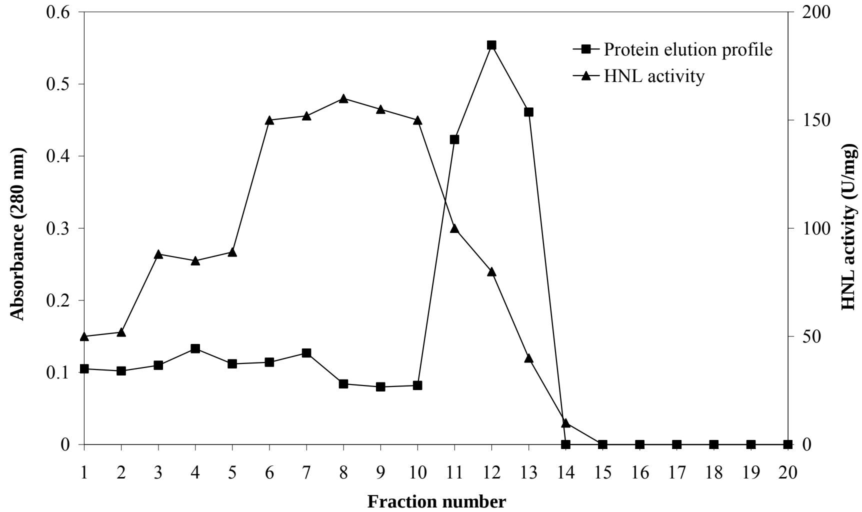

Figure 1: Ion exchange chromatography of crude enzyme preparation on DEAE-Sepharose column (9 x 2 cm, bed volume 25 mL) at a flow rate of 0.5 mL/min and fractions of 4 mL collected each.

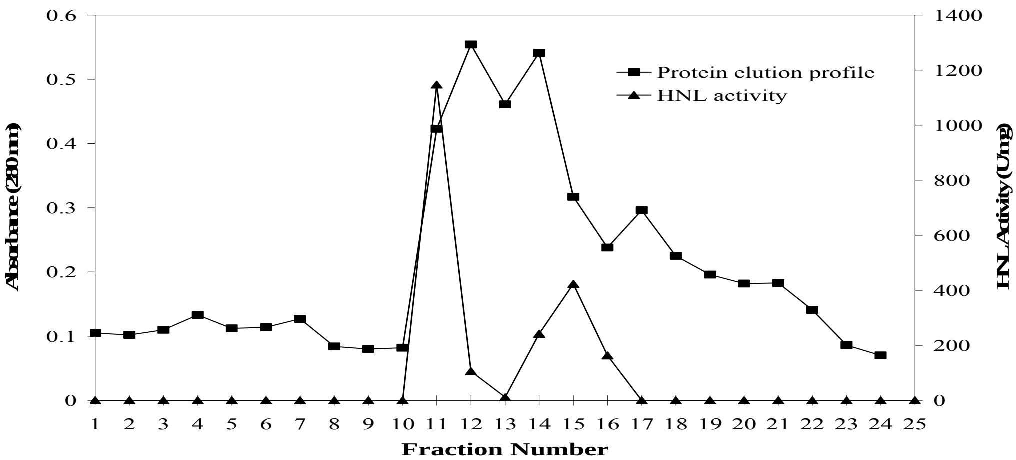

Figure 2: Gel filtration chromatography of DEAE-Sepharose fractions on Sephacryl S-100 column (1.6 x 60 cm) at a flow rate of 0.3 mL/min and fractions of 4 mL collected each.

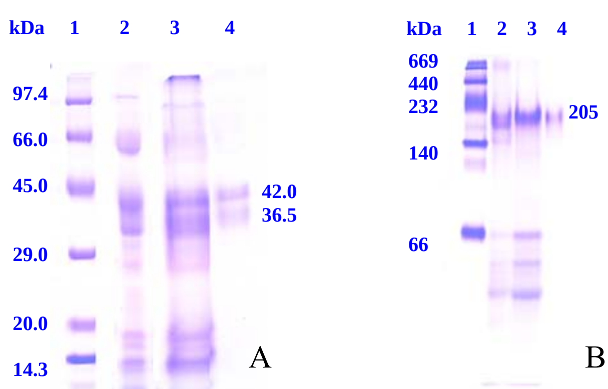

Figure 3: (A) SDS- PAGE of purified hydroxynitrile lyase of rosary pea Lane 1 was loaded with following protein molecular mass standards: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29kDa) and soyabeen trypsin inhibitor (20 kDa), lysozyme (14.3 kDa). Crude enzyme (lane 2), ion exchange chromatography fractions (lane 3) and gel filtration chromatography (lane 4) were applied on to the gel. (B) Native –PAGE of purified hydroxynitrile lyase of rosary pea Lane 1 were loaded with following molecular mass standards: thyroglobuline (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (66 kDa). Crude enzyme (lane 2), ion exchange chromatography fraction (lane 3) and gel filtration chromatography fraction (lane 4) having higher HNL activities were applied on to the gel.

Table 2: Purification of hydroxynitrile lyase of *Abrus precatourius*

Stages of Purifications	Protein mg/ml	Total protein (mg)	Specific activity (units)	Total activity (units)	Yield (%)	Purification (fold)
Crude sample	0.162	24.32	64	1573.2	100	-
DEAE ion exchange	1.34	6.71	150	1016.7	64	2.3
Gel filtration	0.03	0.722	577	416.74	26	9

equilibrated with sodium citrate buffer pH 5. Fraction numbers 11 and 14-16 (Fig. 2) showed high HNL activity**.** In this step, HNL of rosary pea was purified up to 9 fold with a yield of 26 % of enzyme. The fractions having higher HNL activity were pooled. SDS polyacrylamide gel electrophoresis of the purified fractions showed the presence of two distinct protein bands which indicated the heteromeric nature of HNL. The upper band of 42 kDa and lower band of 36.5 kDa were found in SDS-PAGE, whereas the Native-PAGE showed a single band of 205 kDa (Fig. 3 A, B).

Characterization of purified HNL of rosary pea

The different reaction parameters like buffer system, ionic strength of buffer, pH optimization, and reaction temperature were optimized for the purified HNL of rosary pea.

Buffer System

Among the three types of buffers tested, maximum HNL activity (548 Unit/mg) was recorded in 0.1 M, sodium citrate buffer followed by 0.1 M, sodium acetate buffer (295 Unit/mg) and 0.1 M, phosphate buffer (246 Unit/mg) as depicted.

pH optimum

The pH of sodium citrate buffer (0.1 M) was varied from 3.0 to 8.0 in the reaction (Fig. 4). At pH 5 the enzyme exhibited maximum activity (548 Unit/mg). With an increase in pH, a decrease in HNL activity of *Abrus precatorius* was observed.

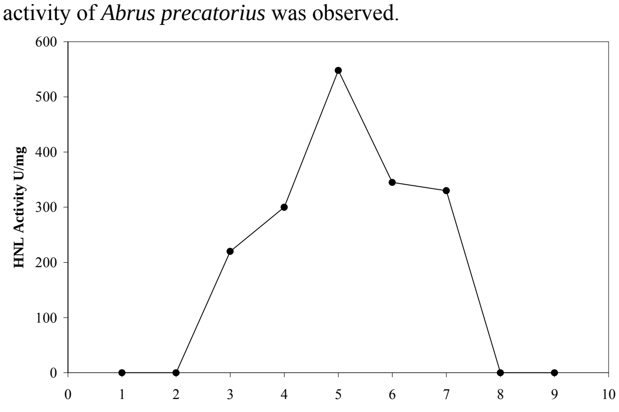

Figure 4: Effect of pH of 0.1 M sodium citrate buffer on HNL activity of *Abrus precatorius.*

Temperature optimum

The activity of purified HNL was assayed at different temperatures (5º-70°C). The optimum temperature for HNL activity was recorded at 30°C (Fig. 5). With an increase in temperature, enzyme showed drastic decrease in its activity. At 45°C or above the enzyme activity was completely lost.

K_M and V_{max} of HNL of rosary pea

A Line Weaver- Burk plot (1/v versus 1/[S]) of velocity data of HNL catalyzed reaction at various concentrations of substrate [S] *i.e.* mandelonitrile was drawn. HNL of *Abrus precatorius* showed a V_{max} of 625µmol/min/mg protein and K_M of 13 mM of mandelonitrile.

Synthesis of cyanohydrins using HNL of rosary pea

The enzymatic reaction using 15 mL (3894.6 Unit) of enzyme and (35 mL) substrate (benzaldehyde) yielded a yellowish (24 mL) oily substance which had density similar to mandelonitrile (Merck). Further the NMR spectroscopy of the product formed was also performed at Punjab University, Chandigarh, which confirms the synthesis of mandelonitrile. The product formed was recemic in nature its steriospecificity was determined by using Crown pack (+) column. In the reaction mixture, 0.147 mole (15 mL) of benzaldehyde was used and finally 0.132 mole (17.5 g) of mandelonitrile was recovered which corresponded to 90 % molar conversion. Yield of the mandelonitrile was 39 g/mg of enzyme used in the reaction mixture.

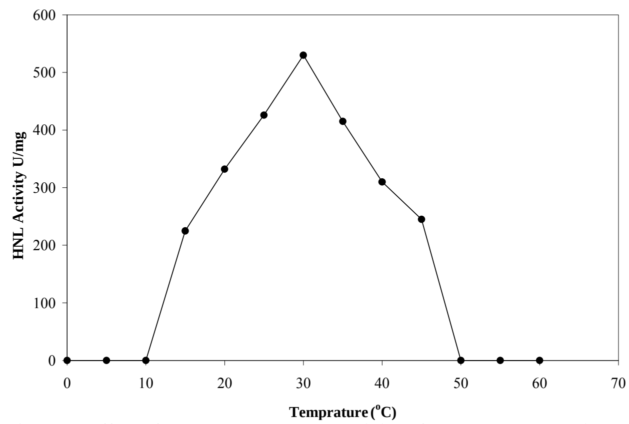

Figure 5: Effect of temperature on HNL activity of *Abrus precatorius* in 0.1 M sodium citrate buffer, pH 5.0.

The major aim of present work was to screen some indigenous plants growing in Himachal Pradesh (Sub Himalayan region) for HNL activity and to find out some new sources of HNL. There were 10 different plant species tested for HNL activity in the present study. Among these 10 plants, *Prunus amygdalus* (Ingrid et al. 2001), *Prunus armeniaca* (Tuncel et al. 1995), *Prunus malus*, *Manihot esculenta* (Kiljunen and Kanerva 1996; Hughes et al. 1994; Jane et al. 1998), *Ficus elastic* (Hasslacher et al. 1996), *Prunus serotina* (Zihua and Poulton 1999) have earlier been reported to possess HNL activity. Out of 10 plants, *Abrus precatorius* emerge as a good source of HNL (577 units/mg of seed protein) in the present study. The HNL activity of *Abrus precatorius* was higher compared to earlier reported work [e.g. 188 units/mg of protein in *Sorghum vulgare* (Seely *et al.* 1966), 111.2 units /mg of protein in *Prunus lyonii* (Xu et al. 1986), 34.1 units/mg protein in *Linum usitatissimum* (Xu et al. 1988) and 258 units/mg in *Prunus amygdalus*] for the source of this enzyme.

HNL of *Abrus precatorius* could be purified in two-steps. The results of purification of HNL from seeds of *Abrus precatorius* showed a 9 fold purification and 26 % of yield. This means that *Abrus precatorius* seeds were rich in HNL proteins comprising about 11 % of total extractable seeds proteins. The recovery (yield) of 26 % of HNL activity at the end of purification showed that there was appreciable loss of enzyme during purification. Such loss in enzyme activity is usually observed during purification (Ho et al. 2004). The low yield of enzyme activity may be due to physical or physiological (inactivation) losses during purification. Since enzyme was fairly stable at 0-4 °C after purification, therefore recovery of 26 % of enzyme activity may be due to physical loss of enzyme during various steps followed for purification. Previously, 4.3 fold purification with 60 % yield (Xu et al. 1986) in *Prunus lyonii* and 1.71 fold purification with 50 % yield in *Linum usitatissimum* (Albrecht et al. 1993), 151 fold purification with 3 % yield (Gray and Conn, 1989) and 122 fold purification with 38 % yield (Albrecht *et al.* 1993). in *Xemia americana* have been reported.

The electrophortic studies (SDS PAGE) of the purified enzyme revealed that HNL of *Abrus precatorius* comprised two polypeptides of 36.5 and 42 kDa. Native PAGE indicated the presence of a single band of 205 kDa (Fig.3 B). This confirms the polymeric in nature of *Abrus precatorius* HNL. Molecular mass diversity of the enzyme has been reported earlier in *Manihot esculenta* (28.5 kDa) (Jane et al. 1998), *Linum usitatissimum* (42 kDa) (Xu et al. 1988), *Prunus lyonii* (59 kDa) (Xu *et al*. 1986), *Phlebodium aureum* (20 kDa) (Wajant et al. 1995), *Prunus serotina* (57 kDa) and in *Phleobodium aureum* (20.1 kDa) (Wajant et al. 1995) using SDS-PAGE.

HNL of *Abrus precatorius* also exhibited some distinct kinetic properties as compared to that of other plants. The K_m and V_{max} values of HNL of *Abrus precatorius* was 13 mM of mandelonitrile as a substrate and 625 µmole/ min/mg of protein respective with mandelonitrile as substrate, whereas K_m and V_{max} of HNL reported from different sources such as *Phleobodium aureum* (0.83 m*M* benzaldehyde and 60.1 µmole/ min/ mg protein) (Wajant *et al.* 1995), *Prunus lyonii* (93m*M* mandelonitrile and 450 µmole/ min/ mg of protein) (Xu et al. 1986) and *Linuum usitatissum* (2.5 m*M* benzaldehyde and 1.11 µmole/ min/ mg of protein). These data indicated that HNL of rosary pea seemed to have fairly higher affinity for mandelonitrile and had higher V_{max} in comparison to earlier reported HNLs. Thus, it holds good potential for its application in the synthesis of cyanohydrins.

The purified enzyme was eventually used in the synthesis of mandelonitrile from benzaldehyde and a significant 90 % molar conversion was obtained. The conversion rate was much higher as compared to previously reported HNLs, e.g. 73 % in case of HNL of *Prunus capuli* and 61 % molar conversion by HNL of *Mammca americana* (Sdis et al. 1998).

Present study revealed that HNL of rosary pea had best activity at 0.1 M of sodium citrate buffer (pH 5). It indicated that HNL was stable and active in acidic conditions. The higher value of V_{max} showed the affinity of HNL towards its substrate i.e. mandelonitrile. The above data confirmed the high potential of rosary pea seeds for large scale production of hydroxynitrile/ cyanohydrins.

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