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Production of Hydrogen Cyanide (HCN) by Purple Non Sulfur Bacterium Isolated from the Rice Field of West Bengal

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Abstract: *Hydrogen cyanide (HCN) is a very common volatile toxic antimicrobial secondary metabolite produced by a number of microorganisms including plant growth promoting rhizobacteria (PGPR) that plays a major key role in plant pathogenic disease suppression through its effective toxicity to the phytopathogens and also serves as an important factor in regulation of phosphorous availability to plants as well as in weed control. A considerable numbers of bacteria produce hydrogen cyanide (HCN) as their volatile or exogenous metabolite secreted in microbial culture. This characteristic can be exploited as a substantial quality to fight against plant and crop pathogens as well as a potential weed growth controlling factor in agricultural fields.*

Aiming the perspective mentioned above, the present work deals with the isolation, purification and identification of a purple non sulfur bacterium (PNSB), tentatively identified as Rubrivivax gelatinosus (strain RASN4) and evaluation of its potentiality for HCN production (in vitro) as a substantial phytopatho-remidiatory quality against a wide range of agricultural crop and plant pathogens and vis-à-vis as an effective measure to control the growth of the weeds in crop fields beside of its other plant growth promoting rhizobacterial (PGPR) features in order to formulate a potential microbial biofertilizer.

For this said purpose, this said purple non sulfur bacterium (PNSB) Rubrivivax gelatinosus (strain RASN4) was first screened qualitatively followed by its quantitative determination of bacterial HCN production (in vitro) in order to confirm this assessment. This is the first time report that the purple non sulfur bacterium (PNSB) Rubrivivax gelatinosus strain RASN4 can produce HCN. Quantitative study showed that the amount of HCN produced by R. gelatinosus (strain RASN4) attended its optimum level on the second day (131µg/ml) through regularly routine examination of the HCN estimation produced exogenously in culture medium up to 15th date of its experimental incubation.

Keywords: Purple non sulfur bacterium, PNSB, Hydrogen cyanide, HCN, *Rubrivivax gelatinosus*.

Introduction

Many bacteria can produce Hydrogen Cyanide (HCN) in form of their volatile or exogenous metabolite in culture medium which can also be exploited for agricultural benefit, as a potential measure against a good number of agricultural plant and crop pathogens as well as also for weed control in agricultural fields.

HCN is a well known broad spectrum volatile microbial secondary metabolite ,much more effectively toxic to plant pathogens (Pal *et.al*., 2000; Voisard *et.al*,1989) produced by a number of plant growth promoting rhizobacteria (PGPR).HCN can play a key role in disease suppression of various crops (Defago *et.al*, 1990, Voisard *et.al*, 1989; Stutz *et.al*., 1986) in addition to their role against soil borne pathogens in order to suppress them (Weller *et.al*.2002; Voisard *et.al*.1989) and also in weed control (Heydari *et.al*.,2015; Kamei, *et.al*.,2014; Kremer and Souissi 2001). HCN plays a significant role in biological control of plant pathogens through the limitations of fungal growth contributing to their antagonism (Ahamad *et.al*.2008; Rezzonico *et.al*.2007; Siddiqui *et.al*, 2006). HCN can also be responsible for suppression of different plant root diseases in tobacco, cucumber and tomato (Flaishman *et.al*.,1996; Ramettee *et.al*.,2003;Voisard *et.al*,1989) as well as causing detrimental effect on many plant pathogenic nematodes (Insunza *et.al*.2002,Gallagher and Manoil 2001). Many plant associated fluorescent pseudomonas have their potentiality for biocontrolling of plant pathogens through their production of several antimicrobial compounds including HCN (Haas and Defago 2005).

Apart from the biocontrol studies, some works also suggested that HCN produced by PGPR plays their significant role in formation of complexes with transitional metals present in minerals (Faramarazi and Brand L 2006; Fairbrother et.al,2009) and also with irons (Keel et.al,1997) and thus reduces the available iron levels for phytopathogens in order to contribute or additional dimension of biocontrol and metabolism of nutrients elements from natural rocky environments (Wongfun et.al.2014;Lapanje et.al,2014;Frey et.al 2010). In addition, HCN also is having an indirect Key role in phosphorous availability and demonstration of metals (Rijavec and Lapanje 2016), Contributing in direct increase of nutrient availability facilitating the enhancement of plant growth and development (Rijavec and Lapanje 2016).

Weed controls at seedling level through the phytoxicity of HCN produced by plant growth promoting rhizobacteria has also been established (Kremer and Souissi 2001). Important role of inhibition potential of rhizobacterial HCN on weed germination (Heydari *et.al*, 2015) and their potential application in Integrated Weed Management (IWM) system, as well as bio pesticides of weeds (Kamei, *et.al*, 2014) also showed a newer approach in modern agricultural practice. Potentiality of microbial production of HCN as their secondary metabolite are very common to the prokaryotes with special reference to the members under phylum Protobacteria including *Pseudomonas aeruginosa* (Blumer and Haas 2002b; Pessi and Haas 2001, 2000; Castric 1981; Wissing 1975, 1974), *Pseudomonas fleuroscence* (Haas and Defago 2005; Blummer and Haas 2002b), *Pseudomonas protegens* (Ramette et.al, 2011), *Chromobacterium violaceum* (Bunch and Knowles 1982).

Recent works supported the view that purple non sulfur bacterial strains have their capability to produce hydrogen cyanide (HCN) as their microbial secondary metabolite. Production of hydrogen cyanide (HCN) by purple non sulfur bacterium *Burkholderia cepacia* was clearly being established in order to substantiate their potentiality with plant growth promoting biocontrol trait (Neerincx, A. H., *et.al*, 2016; Bernier, S. P., *et.al*, 2016; Gilchrist, F. J., *et.al*, 2013; Ryall, B., *et.al*, 2008). Neerincx, A.H. *et.al* (2016) reported that Burkholdaria cepacia has the ability to produce HCN up to 0.35 μLh^{-1} in $9.0x10^9$ CFU.

Batool, K., & Rehman, Y. (2017) reported the capacity of hydrogen cyanide (HCN) production (*in vitro*) by purple non sulfur bacterial (PNSB) strain *Rhodopseudomonas palustris* and *Rhodopseudomonas faecalis* with special reference to their arsenic-redox transformation ability and plant growth promotion activity which was also later being confirmed by Pavitra, (2017) as found in case of two PNSB bacterial strain *Rhodobacter* & *Rhodopseudomonas spp*. with considerable amount of HCN production potentials, isolated from rice rhizospheric soil samples of Karnataka, India.

The purpose of this study has been aimed to explore the potentiality of HCN production (*in vitro*) of purple non-sulfur bacterium (PNSB) Strain RASN4, later identified as *Rubrivivax gelatinosus*, to gain a clearer understanding for the exploitation of this said characteristic feature as an additional phytoremediatory biocontrolling PGPR trait in order to formulate a more potent microbial biofertilizer. Keeping this aim in view, the present work focuses on the isolation, functional characterization, identification, screening

followed by quantification of HCN production under *in vitro* condition by purple non-sulfur bacterial isolate RASN4, isolated from the rhizosphere of a rice field of Hooghly district of West Bengal, India.

Materials and Methods

The specific objective of present work was aimed to gain a better insight for investing the capability of PNSB rhizobacterial isolate strain RASN4 for their assessment of qualitative and quantitative potentiality for synthesizing HCN production capacity under *in vitro* condition. So, the aim of this present investigation has been categorized into following sequential steps of methods:

i) Sampling of soil through the collection of rhizospheric soil samples from the targeted rice fields. ii) Enrichment, Isolation and purification of PNSB bacterial strain from the rice rhizospheric soil samples. iii) Characterization followed by tentative identification of PNSB rhizobacterial Strain, designed as RASN4. (iv) To study *in vitro* HCN production potential of PNSB rhizobacterial isolate Strain RASN4 through screening followed by quantification of HCN production.

Sampling of soil:

The purple non sulfur bacteria (PNSB) were isolated from the rhizosphere soils collected from the rhizosphere of rice fields of Ramnagar (Latitude 22.82 N; Longitude 87.80 E) located in Hooghly district of West Bengal, India. Soil samples were collected aseptically from the rice fields in sterilized containers and transported to the laboratory immediately for bacteriological studies.

Isolation of purple non sulfur bacterium (PNSB):

Isolation of purple non sulfur bacteria (PNSB) was made in modified Biebl and Pfennig's (1981) agar medium in anaerobic condition under continuous illumination (1400$\pm$200 lux) at 32$\pm$2C^{o} (Ponsano,*et.al.*,2003) following a method as described by Archana *et.al.* (2004).

All the bacterial inoculations and incubations used in this experimental study was done strictly under the conditions of bacterial growth as described by Ponsano, *et.al.*, (2003).

Briefly, 1 gram of rhizospheric paddy soil sample was mixed thoroughly with 10 ml of saline water (0.7% NaCL w/v) by vortexing prior to make its tenfold dilutions from which 10^{-5} was used as inoculums in order to pour the same plated with 20 ml of Biebl and Pfennig's (1981) agar medium (40–45 C^{o}) and subjected to their solidification. The inoculated Petri plates were then covered with molten paraffin wax (55–60C^{o}) by over laying method prior to their immediate solidification on pouring over the agar. The petri plates were rotated in a gentle circular motion during pouring of wax in order to spread it uniformly over the agar surface. (Before closing down the lids, the petri plates remained open for 10 min after pouring the molten paraffin wax for the heat radiation (Archana *et.al.*, 2004) and subjected them for incubation in anaerobic condition under continuous illumination (1400$\pm$200 lux) at 32$\pm$2C^{o} (Ponsano, *et.al.*, 2003) with the agar side of the inoculated petri plates remain exposed to the light intensity (Archana *et.al.*, 2004). The overlying paraffin wax layer was totally removed in a gentle manner with the help of an aseptic sterile scalpel after the development of bacterial colonies. Agar embedded bacterial culture colonies were then cut out as sectioned blocks before their transferring aseptically into 15 x 125 mm screw cap tubes completely filled with liquid modified Biebl and Pfennig's (1981) medium prior to their incubation again under continuous illumination (1400$\pm$200 lux) at a 32$\pm$2C^{o} in anaerobic environment for 7 days (Ponsano, et.al.,2003).

The sub cultured bacterial isolates were then purified by streaking them repeatedly on modified Biebl and Pfennig's (1981) agar medium slant prepared in 25x150 mm test tubes, sealed thoroughly for maintaining anaerobic environment under continuous illumination (1400$\pm$200 lux) at a temperature of 32$\pm$2C^{o} (Ponsano,*et.al.*,2003) without altering the other conditions (Ponsano,*et.al.*,2003). The pure sub cultured bacterial colony obtained this way was then aseptically transferred with the help of a sterile aseptic capillary tube into a 125 mm screw cap tube completely filled with Biebl and Pfennig's (1981) medium broth, in order

to incubate the same under anaerobic environment without altering the other conditions (Ponsano,*et.al.*,2003).

The isolated bacterial strain RASN4 was checked for its growth in Nutrient Agar (NA) medium (Atlas, R. M., 2010) and modified RM2 medium (Sinha 1992) respectively under anaerobic environment without altering the other conditions for bacterial growth as described by Ponsano, *et.al.*,(2003) before starting up the main experimentation involving those media.

Purification of purple non sulfur bacterium (PNSB):

It was done by further repetitive streaking on the above medium and finally single bacterial colonies was picked up with the help of sterile aseptic capillary tube and maintained by sub culturing successively in modified RM2 medium (Sinha 1992) in order to get their purest photo heterotrophic growth culture prior to their further characterization and identification.

Characterization and Identification of purple non sulfur bacterium (PNSB):

Purple non sulfur bacterial (PNSB) isolates was characterized through the method of Bergey's Manual of Systematic Bacteriology (1986) followed by automated BIOLOG microbial identification system (Klingler *et al* 1992).

Screening of in vitro hydrogen cyanide (HCN) production potential:

The screening of *in vitro* HCN production ability by the purple non sulfur bacterium (PNSB) Strain RASN4 was performed by the method of Lorck (1948) as modified by Alstrom (1989). The bacterial isolate was sub cultured on Nutrient Agar (NA) medium, supplemented with glycine ($4/4gL^{-1}$). The potential of bacterial HCN production was detected qualitatively after 48 hrs of bacterial inoculation, with the help of a picrate/Na_2CO_3 soaked paper attached to the underside of the lid of the petridishes sealed with parafilm prior to its incubation at $28C^o$ (Lorck 1948).

A change in coloration from yellow to orange-red towards reddish brown of the picrate/Na_2CO_3 soaked paper confirmed the bacterial potentiality of HCN production which was later further reconfirmed by Prussian Blue test as described by Lorck (1948). Briefly, 10 ml of 4N H_2SO_4 were added to a 24 hrs old bacterial culture, followed by distillation of bacterial HCN into a condenser containing 10 ml of 1 N Na_2CO_3. Then addition of 1 drop of 20 percent $FeCl_3$, followed by 2 ml of 10 percent H_2SO_4, and sufficient amount of NaOH was added with 10 ml of the bacterial culture distillate in order to ensure an alkaline reaction for prussian-blue test. A precipitation of prussian-blue coloration confirmed the test after being neutralizing it with HCl confirming the presence of bacterial HCN in culture distillate.

Quantitative estimation of in vitro hydrogen cyanide (HCN) production:

The quantitative assay of bacterial HCN production (*in vitro*) was determined by colorimetric estimation, following the method of Moller and Stefanson (1937) as described by Lorck (1948). Seven consecutive trials were set for this purpose. For each set of trial, the purple non sulfur bacterium (PNSB) Strain RASN4 was sub cultured in 300 ml of conical flasks, each of the 10 flasks contained 50 ml of NA culture medium, supplemented with glycine ($4/4gL^{-1}$), prior to their inoculation at $28C^o$ for 48 hrs under continuous illumination of 1400 ± 200 lux in anaerobic environment (Ponsano, *et.al.*, 2003). After then, the content of the bacterial growth culture from each of the 10 flasks were pooled together, mixed with 10 ml of 4N H_2SO_4 and subjected to vacuum distillation by heating in boiling water bath. 10 ml of distilled material was further mixed with 1N Na_2CO_3 kept in condenser in order to form NaCN, volume which was finally made up to 25 ml by addition of distilled water. The 5ml of distillated NaCN solution were mixed with 10 ml of 1% picric acid in a 25 ml flask and placed in a boiling water bath for 12 mins followed by its cooling. A controlled set was run side by side by mixing 5 ml of distilled H_2O, 2ml of 1N Na_2CO_3 and 10 ml of 1% picric acid. The O.D. was taken at 510 nm and the total amount of cyanide (µg/ml) was estimated with the help of a standard curve prepared with the NaCN (HPLC grade, Sigma).

Results and Discussion:

a) Isolation, Screening and Purification of *R. gelatinosus* Strain RASN4:

Isolation, Screening and Purification of effective PNSB isolate of *Rubrivivax gelatinosus* Strain (RASN4) wa s done after being isolated it from the rice rhizospheric soil of Ramnagar, located at Hooghly district of West Bengal, lndia (Fig:1);which was thoroughly purified prior to their further characterization.

All of the seven replicates of isolated bacterial strain RASN4 were grown up successfully in both of the Nutri ent Agar (NA) (Atlas, R. M., 2010) and modified RM2 medium (Sinha 1992) under the condition of bacterial growth as described by Ponsano *et.al.*, (2003) prior proceeding to further experimentation.

b) Physiochemical characterization of *R. gelatinosus* strain RASN4:

The isolated PNSB bacterial strain RASN4 was first subjected to their normal microscopic study follo wed by Scanning and Transmission Electron Microscopy (SEM & TEM) (fig: 2, 3).

The color of anaerobic bacterial culture ranged from pale peach to purple red in freshly grown conditi on of photo-autotrophic growth under continuous illumination.

All the microscopic studies and measurement of isolated PNSB strain RASN4 revealed that the shape of the individual bacterial cell was curved rod in nature and their size ranged from 0.3-0.7 × 1.3-2.15 µm (wi dth x length).

Their flagellation was polar and monotrichous in nature with positive sign of their motility and slime production as established through further study.

The salt tolerance level of their growth showed at 5% and growth of temperature ranged between 5-4 5 C^o.

The physiochemical characterization, based on the burgey's manual(1986) of isolated *Rubrivivax gela tinosus* strain RASN4 showed positive results to following tests viz tests for nitrogen fixation ability, several enzymatic activities like oxidase, catalase and gelatinase, indole production test, tests for citrate utilization, ni trate reduction, gelatin liquefaction and casein hydrolysis, Huge-Leiffson (O/F) reaction test, bacterial pigme nt study, tests for exopolysaccharide (EPS) production, poly-beta-hydroxy butyrate (PBHB) production, pig ment study of isolated bacterial strain RASN4 established the existence of carotenoid and bacterio-chlorophy ll in their cell. Utilization pattern of different carbon sources, electron donors and growth accilators (by strain RASN4) like acetate, butyrate, citrate, formate, fumarate, glutamate, lactate,malate,propionate,pyruvate,succi nate,tartate;arabinose,cellobiose,fructose,galactose,glucose,lactose,maltose,raffinose,rhamnose,ribose,sucrose ,xylose;glycerol,mannitol and sorbitol were also studied and result was enumerated in Table-1.

Identification of PNSB isolated strain RASN4:

After physiochemical characterisation based on the Burgey's manual (1986) the isolated rice rhizospheric PN SB strain RASN4 was subjected to their confirmed identification up to genus and up to species level through their 16S rRNA analysis followed by BIOLOG^{TM} identification system based on their detailed metabolic fi nger printing involving through utilization patterns of different carbon sources, electron donors and growth accelarators.

From the results, obtained from both of the end, the rice rhizospheric PNSB bacterial strain RASN4 w as confirmly identified as *Rubrivivax gelatinosus* of the family Commamonadaceae that belongs to the order Burkholderelis of beta-proteobacteria group.

In vitro Production of HCN by RASN4 Strain:

The present work established the same said potentiality of *in vitro* HCN production traits in case of PNSB bacterial strain *Rubrivivax gelatinosus* in order to exploit this trait for formulation of a potent microbial biofertilizer with phytopathogenic biocontrol ability.

Assessment of HCN production potential (*in vitro*) through qualitative analysis established the strong indication of *in vitro* HCN production capability of isolated PNSB *Rubrivivax gelatinosus* RASN4 strain which was later been confirmed through its successive quantification.

The isolate *Rubrivivax gelatinosus* RASN4 strain was screened for qualitative detection of their *in vitro* HCN production potentials as summarized in Table-2. A change of the intensity and colour on the filter paper in qu alitative test from yellow to light brown, brown or reddish brown was considered as weak(+), moderate(++) o r strong(+++) indication of reaction respectively (Karmel Reetha *et.al*, 2014). Isolate R. *gelatinosus* RASN4 was proved to be strongly positive for *in vitro* HCN production as indicated by the intensity and change in co loration developed on filter paper (Table-2).

This became further evident from the quantitative study of *in vitro* HCN production (Table-3) of isolated *Ru brivivax gelatinosus* RASN4 strain in comparison to that of the other PNSB bacterial strains having similar p otentiality.

Batool and Rehman (2017) established the potentiality of HCN production by PNSB strain *Rhodopse udomonas palustris and Rhodopseudomonus faecalis* through their work. Among the other PNSB genus, Rho dobacter sp. can also produce significant amount of HCN (Pavitra 2017). *Burkholderia cepacia produce* HC N upto 0.35µlh^{-1} in 9.0 x10^{9} CFU amount (Neerincx *et.al*, 2015).

The results of the quantitative test of *in vitro* HCN production (Table-3) by isolated PNSB *Rubrivivax gelati nosus* strain RASN4 showed significant production of HCN ($p<0.001$) in culture medium. Study of quantitati ve estimation of *in vitro* HCN produced by *R. gelatinosus* RASN4 strain (µg/ml) has been summarized in Ta ble-3 as per daily routine examination. *Rubrivivax gelatinosus* RASN4 showed the maximum amount of HC N production (*in vitro*) on the second day (131µg/ml) through regular examination since their date of incubati on up to the fifteenth date of experimentation (fig.4).

However this is the first time report that the purple non sulfur bacterium (PNSB) *Rubrivivax gelatinosus* strai n RASN4 can produce HCN and the HCN production can reach up to 131 µg/ml level which is much more hi gher than the reported values found in other related microorganisms.

Purple non sulfur bacterium (PNSB) *Rubrivivax gelatinosus* strain RASN4 showed that in *in vitro* culture, th e production of HCN exhibited a bimodal graph with two distinct peaks at 2.62 µg/ml and 2.32 µg/ml on 7th a nd 13th day respectively. However it was intervened by decline in HCN production between 9th and 11th day when it reached 1.37 µg/ml and 1.26 µg/ml concentration level of HCN production respectively.

A likely explanation of this bimodal HCN production showed decline in HCN value on attending the concent ration of 2.62 µg/ml may be either due to inhibitory effect of HCN on the purple non sulfur bacterium (PNSB) *Rubrivivax gelatinosus* strain RASN4 which acted adversely on its metabolic activities resulting in stoppage of HCN production or the HCN concentration might had been induced nitrogenase enzyme (Materassi *et. al.* 1977) which degrade HCN and thus reduced its concentration (1.26 µg/ml - 1.37 µg/ml) which received the i nhibition of HCN production and thereby the concentration of HCN got increased again. The process may be repeated in this ways.

Cyanide is a dreaded toxic antimicrobial secondary biocontrol metabolite, synthesized, excreted and metaboli zed by thousands of microorganisms including bacteria which acts as a general metabolic inhibitor in order t o achieve avoidance of predation or competition without affecting their host plants (Zeller *et.al*, 2007).

Many PGPR are able to produce HCN (Devi *et.al*, 2007) together showing the other PGPR traits in order to a chieve phytoremediatory biocontrol mechanism together with plant growth and development either directly o r indirectly or in a both synergistic way (Joseph *et.al*, 2007). Sometimes PGPR exert their biocontrol ability o n phytopathogens also by some other means like secreating the fungal cell wall degrading enzymes like chiti nase and beta 1,3 glucanase together with the potentiality of HCN secretion as their secondary metabolite (Ch andra *et.al*.2007).

Many plant growth promoting purple non Sulfur bacteria like *Burkholderia cepacia* (Neerinex *et.al*,2016; Be rnier *et.al*,2016; Gilchrist *et.al*,2013;Ryall *et.al*,2008), *Rhodopseudomonous palutris* and *R.faecalis* (Batool and Rehman,2017), *Rhodobacter sp*. (Pavitra 2017) have the ability with considerable amount of HCN produ ction potentials.

Conclusion

In conclusion, it may be summarized that the rice rhizospheric purple non sulfur bacterial Strain, which was i dentified as *Rubrivivax gelatinosus* (RASN4), is capable of showing cyanogenic rhizobacterial traits apart fro m the other plant growth promoting rhizobacterial characteristics. The RASN4 strain has been found to produ ce HCN, a very common volatile antimicrobial secondary metabolite, in considerable amount, that has also b een confirmed through the *in vitro* quantitative determination.

HCN is effectively toxic to the plant pathogens (Pal *et.al* 2002, Voisard 1989) playing a major role in disease suppression (Stutz *et.al*,1986, Voisard.*et.al*.1989,Defago *et.al*.1990,Ramettee 2006) as well as also ha ving their role in regulation of phosphorus availability (Rijavec and Lapanje 2016) and weed control (Kremer and Souissi 2001, Kamei *et.al*,2014,Heydar *et.al*,2015) in addition to their other beneficial PGPR qualities.

This might have been exploited in near future in order to formulate efficient microbial bio fertilizer i noculants with potential phytopatho-remidiatory characteristics, active against a wide range of crop and plant pathogens as well as a strong weed controller in terms of agricultural crop productivity in Indian subcontinen t.

Fig: 1. Map showing location of bacterial sampling site of rice fields at Ramnagar, Hooghly, West Bengal, India (Latitude 22.82 N; Longitude 87.80 E) (Courtesy: Wikimapia)

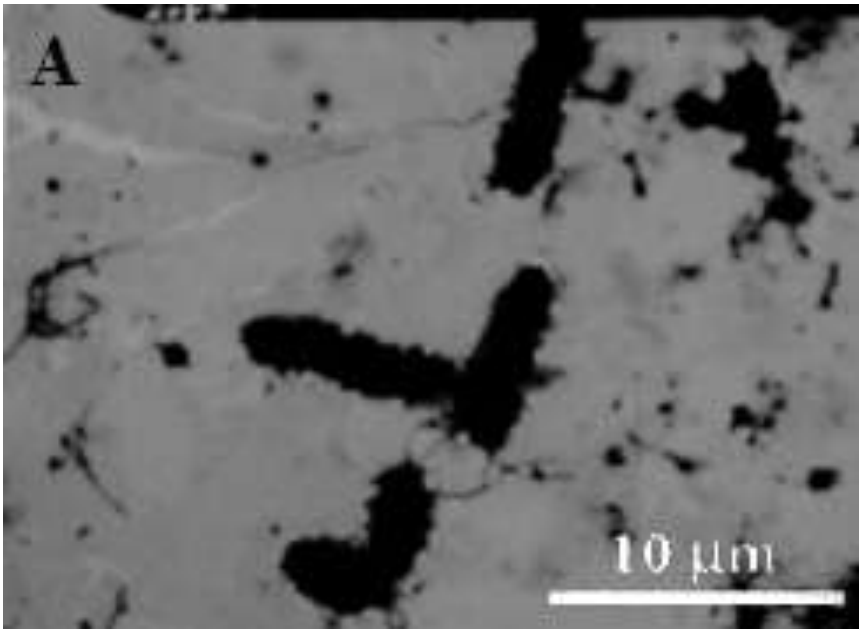

Fig: 2. SEM of *Rubrivivax gelatinosus* Strain RASN4

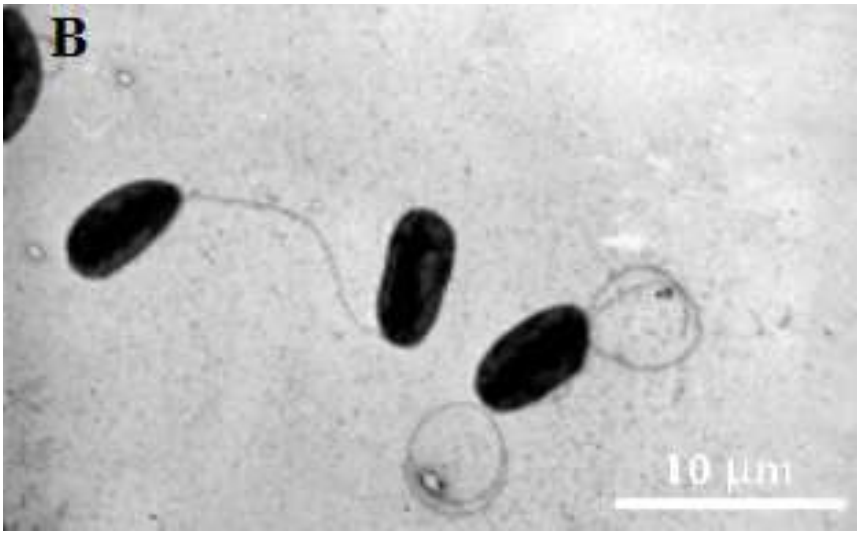

Fig: 3. TEM of *Rubrivivax gelatinosus* Strain RASN4

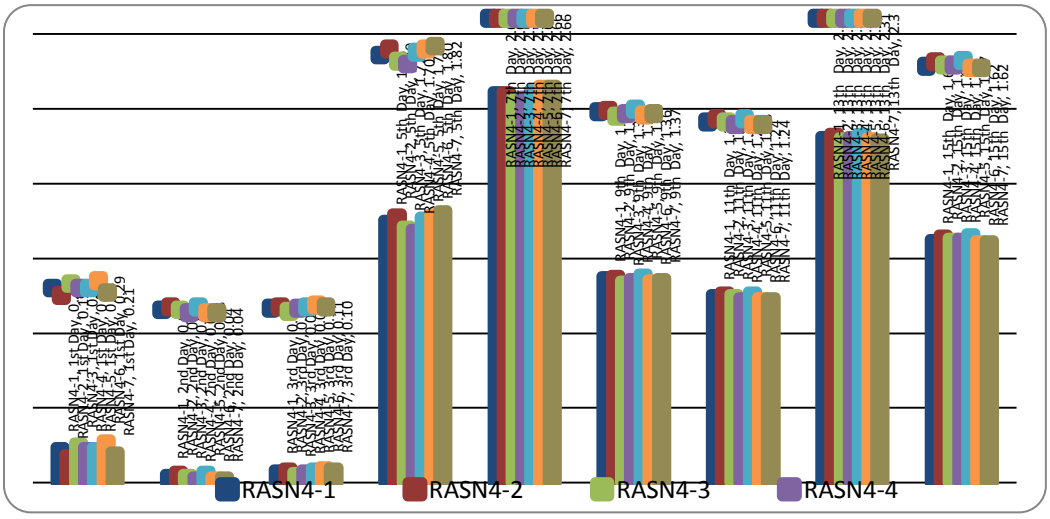

Fig: 4. Bar diagram showing in *vitro* quantitative production (µg/ml) of HCN by PNSB strain RASN4

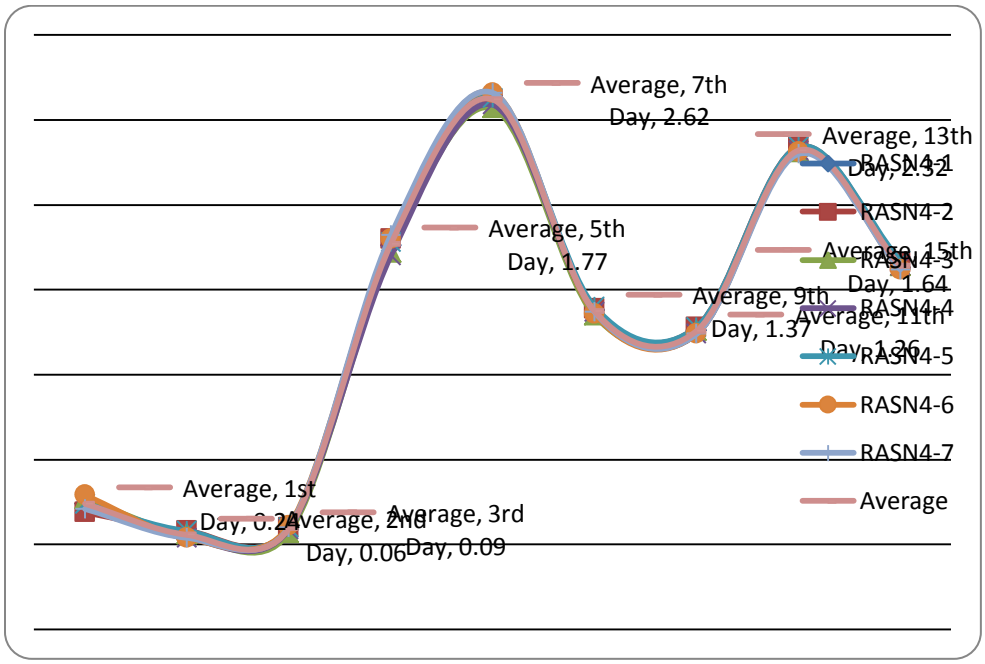

Fig: 5. Graph showing trend of in *vitro* quantitative production (µg/ml) of HCN by PNSB strain RASN4

Table-1: Morphological, physiological and biological characteristics of the isolated bacterial strain *Rubrivivax gelatinosus* (RASN4):

S. No.	Characteristics	Bacterial Isolates *Rubrivivax gelatinosus* (RASN4)
1.	Color of Anaerobic Culture	Pale Peach to Red
2.	Cell Shape	Curved Rod
3.	Size (Width × Length)	(0.3 - 0.7 × 1.3 – 2.1) µm
4.	Formation of Sheaths	-
5.	Gram Staining	Gram negative
6.	Motility	+
7.	Flagellation	Polar, Monotrichous
8.	Slime Production	+
9.	Growth	Photoautotrophic
10.	Salt Tolerance Level	5%
11.	Growth Temperature Range (°C)	5 – 45°C
12.	Nitrogen Fixation Ability	+
13.	Oxidase Activity	+

S. No.	Characteristics	Bacterial Isolates *Rubrivivax gelatinosus* (RASN4)
14.	Catalase Activity	+
15.	Urease Activity	-
16.	NO_3^- Reduction Test	+
17.	Gelatine Liquefaction (Gelatinase)	+
18.	Starch Hydrolysis	-
19.	Casein Hydrolysis	+
20.	**IMViC Test** Indole production Test Methyl Red Test Vogese Proskauer Test Citrate Utilization Test	 + - - +
21.	Hughe-Leiffson (O/F)Reaction Test	O/F
22.	Carotenoid	+
23.	Bacteriochlorophyll	+
24.	Exo-Polysachharides (EPS)	+
25.	Poly-Beta-Hydroxybutyrate (PBHB)	+
26.	Growth Accelerators	Biotin, Thiamine

S. No.	Characteristics	Bacterial Isolates *Rubrivivax gelatinosus* (RASN4)

27.	**Utilization of Carbon Source**	
	Acetate	+
	Butyrate	ND
	Cytrate	+
	Formate	+
	Fumarate	+
	Glutamate	+
	Lactate	+
	L-Malate	+
	Propionate	+
	Pyruvate	+
	Succinate	+
	Tartate	+
	Arabinose	-
	Cellbiose	+
	Fructose	ND
	D-Galactose	+
	Glucose	+
	Lactose	-
	Maltose	-
	Raffinose	-
	Rhamnose	ND
	D-Ribose	+
	Sucrose	+
	Xylose	-
	Glycerol	-
	Mannitol	-
	Sorbitol	

+ Indicates presence of positive reaction; - Indicates absence or negative reaction; O = Oxidation; F = Fermentation;
ND = Not Determined.

Table-2: Qualitative detection of HCN production (*in vitro*) by isolated bacterial strain *Rubrivivax gelatinosus* (RASN4):

Isolate	HCN Production [a] (in vitro)
Rubrivivax gelatinosus RASN4-1	+++[bc]

Rubrivivax gelatinosus RASN4-2	+++[bc]
Rubrivivax gelatinosus RASN4-3	++[bc]
Rubrivivax gelatinosus RASN4-4	+++[bc]
Rubrivivax gelatinosus RASN4-5	+++[bc]
Rubrivivax gelatinosus RASN4-6	++[bc]
Rubrivivax gelatinosus RASN4-7	+++[bc]
Control	---

[a] Intensity of HCN reaction with picrate indicator: none - ; Weak, + ; moderate, ++; Strong, +++

[b] Reaction detectable after at 48 h of HCN initiation assay.

[c] Further confirmed through Prussian blue test.

Table-3:

Strain \ Day	1st Day	2nd Day	3rd Day	5th Day	7th Day	9th Day	11th Day	13th Day	15th Day
RASN4-1	0.24	0.06	0.09	1.76	2.62	1.38	1.26	2.32	1.63
RASN4-2	0.19	0.08	0.10	1.80	2.62	1.39	1.28	2.34	1.66
RASN4-3	0.27	0.06	0.07	1.72	2.57	1.35	1.26	2.31	1.64
RASN4-4	0.24	0.04	0.09	1.70	2.59	1.37	1.24	2.32	1.64
RASN4-5	0.24	0.08	0.10	1.78	2.63	1.40	1.28	2.34	1.67
RASN4-6	0.29	0.04	0.11	1.80	2.66	1.36	1.24	2.31	1.62
RASN4-7	0.21	0.04	0.10	1.82	2.66	1.37	1.24	2.3	1.62
Average	0.24	0.06	0.09	1.77	2.62	1.37	1.26	2.32	1.64
Min	0.19	0.04	0.07	1.70	2.57	1.35	1.24	2.30	1.62
Max	0.29	0.08	0.11	1.82	2.66	1.40	1.28	2.34	1.67
S.D.	0.034	0.018	0.013	0.045	0.033	0.017	0.018	0.015	0.019
S.E.	0.013	0.007	0.005	0.017	0.013	0.006	0.007	0.006	0.007

Quantitative detection of *in vitro* HCN production (µg/ml) by isolated bacterial strain *Rubrivivax gelatinosus* (RASN4): ($p<0.001$)

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