



SCREENING OF ORGANIC ACID PRODUCTION POTENTIALITY OF A RICE RHIZOSPHERIC PURPLE NON SULFUR BACTERIUM *RUBRIVIVAX GELATINOSUS* STRAIN RASN4

Debamalya Gupta¹, Ranjini Chakraborty², Humayun Kabir⁵, Susmita Halder¹, Sayan Choudhary³, Karabi Biswas¹, Jayanta Chowdhury⁴, Astom Mondal¹, Snigdha Majumdar¹, Momezul Haque¹, Sushree Ghosh¹ Ayon Banerjee¹ and Sankar Narayan Sinha^{1*}

¹Environmental Microbiology Research Laboratory, Department of Botany, University of Kalyani, India.

²Department of Biological Sc., MHMS, Chandi Ghosh Road, Kolkata-70040, West Bengal, India.

³Directorate of Open and Distance Learning, University of Kalyani, West Bengal, India.

⁴Department of Microbiology, Techno India University, EM-4, Sector-V, Salt Lake, Kolkata-70009, India.

⁵Quality Assurance Laboratory, Fresenius Kabi Oncology Limited, Kalyani-741235, West Bengal, India.

*Corresponding author

ABSTRACT

Organic acids are long chained organic carbon compounds with their attached functional carboxylic groups showing acidic properties, produced exogenously by a number of microorganisms including plant growth promoting rhizobacteria (PGPR) showing along with their other PGPR potential traits. This characteristics can be exploited as a potentially significant quality for promoting of plant growth, development and vigor with substantial enhancement of crop productivity and yield by facilitating microbial biomineralization of various insoluble inorganic macro and micro trace metal elements that are essential for plants, by converting them into their solubilised plant available forms in order to facilitate plant growth and development. So, such potentiality of exogenous organic acid bio production could be of an another advantageous capability for PGPRs which has its additional synergistic significance along with the other common traditional PGPR traits of PGPR microorganisms in order to bioformulate a potential microbial biofertilizer for agricultural crop fields.

For this said purpose, the isolated Purple non sulfur bacterium (PNSB) *Rubrivivax gelatinosus* strain (RASN4) was first screened qualitatively in terms of their comparative *Rf* values with reference control of known specific organic acids using as control on paper chromatography followed by their determination of acid unitage (AU) values. The *Rf* values obtained from the bacterial culture supernatant of isolated RASN4 strain on paper chromatography were found as 0.66, 0.92, 1.03, 0.12, 0.23, and 0.34 respectively that were compared with their specific reference control of known specific organic acids, and found to be coincided with citric (0.72), fumaric (0.98), succinic (1.08), acetic (0.17), propionic (0.27) and butyric (0.37) respectively. This has become further evident from the determination of acid unitage (AU) values of the isolated strain RASN4 culture supernatant with the highest value of 3.11 (4th replicate) and lowest values of 2.88 (5th replicate) with an average (AU) value of 2.96. So after obtaining the results from the both end followed by their comparing, it can be postulated that the rice rhizospheric PNSB bacterium *Rubrivivax gelatinosus* (RASN4) has the significant potentiality for showing promising performance for microbial organic acids exoproduction along with its other

traditional PGPR traits. This is also the first time report of organic acid exoproduction by PNSB bacterial strain *Rubrivivax gelatinosus* (RSN4) as became evident through qualitative screening of the present study that can be exploited to bioformulate a potential microbial bio fertilizer for the benefit of agriculture and mankind .

Keyword: Purple non sulfur bacterium, PNSB, organic acid production, *Rubrivivax gelatinosus*

INTRODUCTION

Organic acids comprise of a long chain of carbons with its attached carboxylic group. (Shaikh and Qureshi 2013) postulated that organic acids are mostly the organic carboxylic compounds with acidic properties associated with their functional carboxylic group (-COOH).

The microbial world comprises of widely diverse type of microorganism including bacteria with their similarly diversified metabolic activities resulting into production of a wide range of different products; many of which are organic acids expected by the bacterial microorganisms (Khan and Gupta, 2015). Many anaerobic and facultative anaerobic micro organisms can produce a range of organic acids including lactic, succinic, acetic, citric, butyric and propionic acids (Gottschalk 1985). The gram positive facultative anaerobic bacteria produce organic acids after growing on sugars as carbon sources (Nishimura *et.al*, 2007); Schaffer and Burkovski 2005; Takeno *et.al*, 2007). Types of organic acid produce and their production amount may also differ with different micro organisms. Shaikh and Qureshi (2013) screened the isolated organic acid producers from different samples of diversified habitats. Khan and Gupta (2015) screened and optimized the organic acid producing micro organisms with special reference to fungi of the Chattisgarh mine area of India.

A wide range of soil micro organisms including bacteria and fungi of plant rhizospheric soil release organic acids. There had been many earlier reports on secretion of organic acids in the rhizospheric soil as a result of the activities of rhizospheric microbial consortium (Schwartz *et.al.*, 1954; Sperber 1958; Subramanyam 1929). Sperber (1958) studied the production of organic acid potentials of soil micro organisms from the view point of their role in phosphate solubilisation. Chandrasekaran (1969) assessed the potentiality of organic acid production by the soil micro organisms of agricultural rhizospheric soil and found soil rhizospheric bacteria were with a greater potential of organic acid production in comparison to that of the non rhizospheric ones. In addition, the rhizospheric micro organisms with legumes could produce a greater amount of organic acids than those same, associated with non legumes (Chandrasekaran; 1969). Among the soil micro organisms, the plant growth promoting rhizobacteria (PGPR) plays a major key role in plant growth and development along with the enhancement of their crop productivity through the microbial biosolubilization of macro and micro trace element metal by converting them from their inorganic unavailable form to solubilized bio available form (vidyashree *et .al.*,2018).

The chief reasons behind this rhizobacteria mediated mineral bio solubilization are attributed to production of various organic acids and related pH drop by the plant rhizospheric microbial consortium (Alexander, 1997). Micro organisms absorb NH_4^+ ions and thus influence the rhizospheric soil pH in order to eject protons to compensate the membrane potential difference and acidify the soil rhizosphere. Moreover, presence of CO_2 liberated in both root and through microbial respiration also contributes directly into acidification (Jones and Oburger, 2011). Lowering of pH by micro organisms, carbon and nitrogen sources can also play as sustainable factors in organic acid production (Di Simone *et.al.*, 1998; Reyes *et. al*, 1999).

Soil micro organisms can also synthesize organic acids in rhizospheric environment as an adaptation mechanism in various biotic and abiotic stress responses (Brimecombe et. al, 2001; Yates and Morgan 2005) irrespective of the osmotic potential difference (Jones and Brassington 1998). Micro organisms produce a wide range of organic acids after consumption of plant root exudates specially under nutrient limiting situations (Rozycki 1985; Rozycki and Strzelczyk 1986).

Crowly et .al (1991) and Von Wiren et. al, (1995) showed the microbial effects on phytometallophore efficiency. Many micro organisms are capable of using organic acid-metal complexes as well as non complexed free organic acids (Bergsma and Konings, 1983).

Jones et.al (1996) established that soil bacteria can regulate the type and amount of transporters involved in organic acid uptake into cells on the basis of their carbon supply availability.

Secretion of organic acids in large amount into soil rhizosphere not only facilitate the pre existing rhizobacterial growth but also serve as chemo attractants for induction of bacterial chemotactic movements towards plant roots, specially as found in case of flagellate bacterial and fungal hyphae even at a very low concentration of 10uM as shown by experimentation in order to induce the said chemotactic response, which is similar to the concentration of rhizospheric soil solutions (Barbour et. al 1991; Shen et.al, 1996, Zheng and Sinclair 1996).

Organic acids from microbial origin also serves as the carbon sources for microorganisms during the time of solubilisation of phosphorous which gets consumed and reduced as a result of which, the solubilising effects provide the opportunity to estimate the life span of those organic acids between 0.5 and 12h. This postulates that secretion of those organic acids must be of a continuous process that secreted downwards (Xiao and Wu 2014).

Many gram negative bacteria can produce gluconic acid from glucose through direct periplasmic oxidation of glucose which is postulated as the metabolic basis of their inorganic phosphate solubilisation as competitive strategy of transformation of readily available carbon sources into less ready to utilize products by other microorganisms (Goldstein 1995; Whiting et.al.,1976).

Similarly, oxalic acids of microbial origins are thought to be involved in a number of metabolic processes like formation of free radicals, extra cellular pH modification, precipitation of calcium followed by pectin hydrolysis and acid catalysis of hemicelluloses and cellulose apart from their roles in phosphorous and iron acquisition (Green and Highley 1997; Micales 1997).

The purposes of these organic acids secretion by microbes are chiefly attributed to make various insoluble mineral elements of soil, being made available to the plants in their soluble form (Morgan et.al, 2005; Balogh-Brunstad et.al, 2008).

The potentiality of microbial organic acids for metal solubilization has opened up a new area for their application for metal leaching from low grade mineral ores in the field of mineral biotechnology (Joshi and Luthra 2000).

Microbes can convert metal compounds into their water soluble bio available forms and can act as biocatalysts of those leaching processes (Acharya 1990).

Vidyashree et.al, (2018) studied the potentiality of organic acid production by microorganisms aiding in Zinc solubilization. Plants can uptake zinc from rhizospheric soil as divalent ion of zinc (Zn^{2+}) by releasing the cations through the action of organic acids excreted in the rhizospheric environment by the micro-organisms that plays a pivotal mechanism in Zinc bio solubilization (Vidyashree et.al,2018).

Additionally, the anions can increase Zinc solubility through chelation of Zinc in order to convert them in their enhanced bio available form (Zn^{2+}) available to the crop plants (Jones and Darrah, 1994).

Microbial Conversion of inorganic insoluble phosphorous into their soluble bio available form makes insoluble forms of phosphorus readily available to the plants as a result of which, increase in soil fertility and crop productivity in association with high social, economical and environmental benefits also gets achieved (Serna passo et,al,2017).

Gerretsen (1948) reported that plants being inoculated with soil microorganisms, show their improved growth with increased phosphate uptake from their insoluble forms which was later also being confirmed by the other workers (Pikovskaya, 1948); Menkina 1950; Manzons and Tomasher'ska, 1956).

Phosphate solubilizing bacterial microorganisms (PSB) can solubilize inorganic insoluble mineral phosphates into their bio available soluble forms by the production of organic acids like citric or succinic acid etc. (Rodriguez and Fraga 1999; Sharma et, al, 2013).

PSB utilizes such soluble bio available form of inorganic phosphates for their own growth and the rest surplus amount of that same is absorbed by the plants that ultimately results into enhancement of their growth and development. When such PSB like *Pseudomonas*, *Bacillus* were applied to the crop plants like barley and potato, it led to increased grain and biomass yield with better plant growth and development (Sahin et, al., 2004; Malboobi et, al., 2009).

PSB strains from different bacterial genera like *Pseudomonas*, *Rhizobium* and *Enterobacter* are of the efficient producers of organic acids in soil environment (Rodriguez and Fraga 1999, Whitelaw, 2000).

Production of organic acids by bacterial microorganisms in context to inorganic insoluble mineral phosphate solubilisation becomes a matter of interest for so many workers as their prime object of research.

Gerretsen (1948) and Pikovskaya(1948) observed improvement of growth with increased phosphate uptake from their inorganic forms by the plants, inoculated with soil microorganisms. This was also confirmed later by the other workers (Menkina 1950; Manzons and Tomasher'ska, 1956).

Many workers observed application of Phosphate solubilizing bacteria (PSB) along with VAM and rock phosphate showed an increase in phosphorus nutrition of plants in alkaline soils (Bolan et,al.,1987, Omar 1997;Piccini and Azcon 1987;Vassileva et,al,1998). Rodriguez and Fraga (1999) studied the potentiality of PSB for solubilizing insoluble inorganic mineral phosphate into their bio available soluble organic forms through the microbial production of different types of organic acids.

Gyaneshwar et,al.,(1998) demonstrated bacterial potentiality for solubilizing rock phosphates through secretion of a mixture of different organic acids at a very low concentration (less than 5 mM).

Gadd (1999) and Vassilev et.al. (1996) studied the activities of microorganisms with potentiality of organic acid secretion in order to solubilize rock phosphates into their soluble bio-available forms for plants. Whitelaw (2000) also confirmed the capability of different PSB microorganisms for their efficient production of organic acids in soil environment. Srivastava et,al.,(2003) investigated the effects and efficacy of PSB microorganisms in solubilization of phosphates and rock phosphates of acidic alfisol through their organic acid secretion potentials (Srivastava et,al.,2003).

Bacteria	Organic Acid Produced	Reference
<i>Gluconobacter oxydans</i> , <i>Pseudomonas</i> and <i>Acetobacter</i> <i>spp.</i>	Gluconic acid	(Ramachandran et al., 2006).
<i>Gluconobacter spp</i>	Gluconic acid	(Kubicek et al., 2010).
<i>Anaerobiospirillum succiniproducens</i> , <i>Actinobacillus succinogenes</i> , <i>Bacteroides fragilis</i> and <i>Mannheimia succiniproducens</i> .	Succinic acid	(Leng et al., 1967).
<i>Corynebacterium glutamicum</i>	Succinic acid	(Okino et al., 2005, 2008).
<i>Corynebacterium glutamicum</i>	lactic acid	(Okino et al., 2005).
<i>Actinobacillus succinogenes</i>	Succinic acid	(Guettler et al., 1999), (McKinley and Vieille, 2008).
<i>Mannheimia succiniproducens</i>	Succinic acid	(Lee et al., 2002).
<i>Mannheimia succiniproducens</i>	lactic acid	(Song et al., 2007a, b).
<i>Mannheimia succiniproducens</i>	Acetic acid	(Song et al., 2007a, b).
<i>Mannheimia succiniproducens</i>	Formic acid	(Song et al., 2007a, b).
<i>Anaerobiospirillum succiniproducens</i>	succinic acid	(Davis et al., 1976),(Glassner and Datta, 1992), (Meynial-Salles et al., 2008).
<i>Brevibacterium ammoniagenes</i>	Fumaric acid	(Goldberg et al., 2006). (Giorno et al. 2001)
<i>Bacterium xylinoides</i> ,	Kojic acid	(Wilson, 1971).
<i>Glucono-acetobacter opacus var.</i> <i>mobilis</i>	Kojic acid	(Wilson, 1971).
<i>Gyrinium roseum</i>	Kojic acid	(Wilson, 1971).
<i>Lactobacillus rhamnosus</i> , <i>Lactobacillus spp.</i>	Lactic acid	Hofvendahl, K., & Hahn-Hägerdal, B. (2000).
<i>Lactobacillus amylophilus</i> ,	Lactic acid	Altaf, M. D., Naveena, B. J., & Reddy, G. (2007).
<i>Lactobacillus delbrueckii</i>	Lactic acid	John, R. P., Nampoothiri, K. M., & Pandey, A. (2007).
<i>Actinobacillus succinogenes</i>	Succinic acid	Du, C., Lin, S. K. C., Koutinas, A., Wang, R., & Webb, C. (2007).
<i>Mannheimia succiniciproducens</i>	Succinic acid	Lee, S. J., Song, H., & Lee, S. Y. (2006).

<i>Escherichia coli</i>	Succinic acid	Lin, H., Bennett, G. N., & San, K. Y. (2005).
<i>Actinobacillus succinogenes</i>	Succinic acid	Liu, Y. P., Zheng, P., Sun, Z. H., Ni, Y., Dong, J. J., & Zhu, L. L. (2008).
<i>Anaerobiospirillum succiniciproducens</i>	Succinic acid	Meynial-Salles, I., Dorotyn, S., & Soucaille, P. (2008).
<i>Carnobacterium funditum</i> <i>Carnobacterium alterfunditum</i>	Lactic acid	Franzmann et al. 1991
<i>Lactobacillus plantarum</i>	Lactic acid	Fu Wenge 1999
<i>Lactococcus sp.</i>	Lactic acid	Roissart 1994;
<i>Leuconostoc mesenteroides</i>	Lactic acid	Fitzpatrick and Keefe 2001
<i>Enterococcus faecium</i>	Lactic acid	Nolasco-Hipolito et al. 2012
<i>Escherichia sp.</i>	Lactic acid	Maas et al. 2008
<i>Lactobacillus pentosus</i>	Lactic acid	Garde et al. 2002
<i>Arthrobacter paraffinens</i>	Citric acid	Kroya Fermentation Industry 1970
<i>Bacillus licheniformis</i>	Citric acid	Kroya Fermentation Industry 1970
<i>Corynebacterium sp.</i>	Citric acid	Sardinas 1972
<i>Aerobacter sp.</i>	Citric acid	Fukuda et al. 1970
<i>Pseudomonas sp.</i>	Citric acid	Kapoor et al. 1983
<i>Actinobacillus succinogenes</i>	Succinic acid	Li et al., 2011, Xi et al. 2012
<i>Anaerobiospirillum succiniciproducens</i>	Succinic acid	Lee et al. 2001; Jabalquinto et al. 2004
<i>Mannheimia succiniciproducens</i>	Succinic acid	Lee et al. 2003; Beauprez et al. 2010
<i>Bacillus fragilis</i>	Succinic acid	Okino et al. 2005
<i>Corynebacterium glutamicum</i>	Succinic acid	Khan et al. 2009
<i>E.coli</i>	Succinic acid	Chatterjee et al. 2001
<i>Carnobacterium funditum</i>	lactic acid	Franzmann et al. 1991
<i>Carnobacterium alterfunditum</i>	lactic acid	Franzmann et al. 1991
<i>Lactobacillus plantarum</i>	lactic acid	Fu, W., & Mathews, A. P. (1999)
<i>Lactococcus sp</i>	lactic acid	Roissart 1994; Akerberg et al. 1998
<i>Leuconostoc mesenteroides</i>	lactic acid	Fitzpatrick and Keefe 2001
<i>Enterococcus faecium</i>	lactic acid	Nolasco-Hipolito et al. 2012
<i>Escherichia sp.</i>	lactic acid	Maas et al. 2008

<i>Lactobacillus pentosus</i>	lactic acid	Garde et al. 2002
<i>Pseudomonas sp.</i>	Lactobionic acid	Miyamoto, Y., Ooi, T., & Kinoshita, S. (2000).
<i>Pseudomonas fragi</i>	Lactobionic acid	Stodola and Lockwood 1947
<i>P. quercito-pyrogallica</i>	Lactobionic acid	Masuo et al. 1952
<i>Acetobacter orientalis</i>	Lactobionic acid	Kiryu et al. 2012
<i>Burkholderia cepacia</i>	Lactobionic acid	Murakami et al. 2006
<i>Halobacterium saccharovororum</i>	Lactobionic acid	Tomlinson et al. 1978
<i>Bacterium anitratum</i>	Lactobionic acid	Villecourt and Blachere 1955
<i>Paraconiothyrium sp.</i>	Lactobionic acid	Murakami et al. 2008
<i>Zymomonas mobilis</i>	Lactobionic acid	Satory et al. 1997
<i>Pseudomonas ovalis</i>	gluconic acid	Bull et al. 1970
<i>Acetobacter methanolicus</i>	gluconic acid	Loffhagen et al. 1993
<i>Acetobacter suboxydans</i>	gluconic acid	Currie and Carter 1930
<i>Zymomonas mobilis</i>	gluconic acid	Kim et al. 1992
<i>Acetobacter diazotrophicus</i>	gluconic acid	Attwood et al. 1991
<i>Gluconobacter oxydans</i>	gluconic acid	Velizarov et al. 1994
<i>Gluconobacter suboxydans</i>	gluconic acid	Shiraishi et al. 1989
<i>Azospirillum brasiliense</i>	gluconic acid	Rodriguez et al. 2004
<i>Pseudomonas sp.</i>	Fumaric acid	Otsuka 1961
<i>Alcaligenes faecalis</i>	Fumaric acid	Takamura et al. 1969
<i>Pseudomonas alcaligenes</i>	Fumaric acid	Nakajima Kambe et al. 1997; Ichikawa et al. 2003
<i>Pseudomonas fluorescens</i>	Fumaric acid	Nakajima Kambe et al. 1997; Ichikawa et al. 2003
<i>Clostridium propionicum</i>	Propionic acid	Cardon and Barker 1947
<i>Propionibacterium freudenreichii</i>	Propionic acid	Playne 1985
<i>Propionibacterium acidipropionici</i>	Propionic acid	Woskow & Glatz 1991
<i>Propionibacterium jensenii</i>	Propionic acid	Zhuge et al. 2013
<i>Komagataeibacter europaeus</i>	Acetic acid	Vegas et al. 2010, Hidalgo et al. 2012, 2013
<i>Acetobacter lovaniensis</i>	Acetic acid	Gullo et al. 2009

Vyas and Gulati (2009) aimed their work to explicate bacterial organic acid production during inorganic phosphate solubilization and also evaluated their effects on plant growth promotion through the function of inorganic phosphate solubilization into a soluble bio available form, easier for plant uptake.

A summary of organic acid producing bacterial microorganisms can be schematically enumerated as follows

Serena Posso et.al.,(2017) evaluated qualitatively and then through its quantitative assessment of organic acid production and formation of soluble phosphorus by metabolic activity of PSB microorganisms collected from typical Melanudands of Colombia.

Saikia (2018) studied the organic acid production potentiality by PSB microorganisms isolated from agricultural fields and also substantiated their role in mineral phosphate solubilization through the assessment of their capability of organic acid production.

Bacteria can employ a number of ways for bio solubilization of insoluble inorganic mineral phosphates in order to make them available for the plant kingdom for facilitation of plant growth and development. Scervino et.al.,(2010) postulated that production of bacterial organic acids also got influenced by the microorganisms and source of phosphorus where the bacteria grow. The ketoglutaric acid of microbial origin could mobilize small amounts of soil phosphorus, albeit only at a very high concentrations (Moghimi et.al., 1978).

Organic acids are the sources of biotically generated H^+ ions that can facilitate mineralization of bound soil phosphates in order to make them available for up taking of plants (Bhattyacharya and Jain, 2000). Mahdi et.al.,(2011) established through their observation that tri and di carboxylic acids were much more effective in comparison to monobasic and aromatic acids aliphatic acids for facilitating phosphorus solubilization.

The solubilization of different mother sources of soil phosphorus depends upon the types of acid produced together with its total amount of production (Cunningham and Kuiack 1992; Kang et.al., 2002).

This also involves different types of mechanisms through which the inorganic insoluble phosphorus gets transformed into their biologically available soluble forms.

When the mother sources of phosphorus are organic materials and humus, the chief mechanism of phosphorus solubilization by soil microorganisms is mostly attributed to different types of phosphatase enzyme production to carry out the bio mineralization process in order to make it biologically available to the plants and microorganisms (Jones and Oburger 2011).

When phosphorus is trapped within inorganic molecules, the key mechanism of phosphorus solubilization by soil microorganisms is mineral dissolution property related solubilization process, also related to the same of medium acidification.

Release of phosphorus from the insoluble inorganic phosphates by the secretion of organic acids and chelating capacity of many soil microorganisms were established (Goldstein 1995, Kim et.al.,1998,Chen et.al.,2006).

Organic acids of microbial origin including gluconic, citric, malic, Malonic, oxalic, succinic, Lactic and Tartaric acids provide both proton and organic anions that can act as potential chelating agents.

Such negatively charged anions have their ability of formation complexes with positively charged mineral cations like Ca^{+2} , Al^{+3} , Fe^{+3} in soil as a result of which phosphorus gets released on the other hand (Mardad et.al.,2013). Such organic acids can form metal complexes by reacting with metals like aluminum, calcium and iron and thereby release the phosphorus trapped in the respective metalophosphates into soil and also can increase the phosphorus availability through the blockage of phosphorus adsorption sites of soil particles (Jones 1998,Rodrigues and Fraga 1999;Gyaneswar et.al.,2002).

Production of organic acids with low molecular weight by microorganisms facilitate the enhancement of mobilization of phosphate compounds due to the characteristically low concentration (ranging from 1 to 50 mM) of organic acids present in soil solution (Strobel 2001;Goldstein,1995;Bolan et.al.,1994).

Low molecular weight organic acids can effectively solubilize phosphorus trapped in aluminum phosphate and ferric phosphate by chelating iron (Fe) and aluminium (Al) in a very efficient way

(Bolan et.al., 1994; Gilroy and Jones 2000; Jones and Darrah 1994; Kpombrekou-a and Tatabai 1994).

Microbial production of organic acid is a promising approach in recent day's biotechnology (Sauer et.al, 2008). Production of organic acid through an economically commercial process open up new markets by providing new opportunities for the chemical industries (Sauer et.al, 2008). A rising chemical segment of industrial production including fumaric, propionic and itaconic acids are chiefly attributed to organic acid production through microbial fermentation of carbohydrates and alike substrates (Jiang et.al.,2012).

Organic acids have their commercial usages in food technology and beverage industries as well as for the other purposes including as blood anticoagulant, in pharmaceuticals, in cosmetic industries and also as metal cleaning agents together with their other usages in plastic industries, in dyeing and calico printing ,as silvering agents, and in manufacturing engraving inks (Jongh 2006, Magnuson et.al.2004).

In chemical industries organic acids of microbial origin are used in various polymer and solvent production processes (Naraian and Kumari, 2017). In food processing industries organic acids are utilized for addition of flavor, as antioxidant and for maintaining organoleptic properties (Gurtler and Mai 2014). They are also being utilized for food preservation and as food additives for extending self-life of the perishable food ingredients by preventing their deterioration (Cherrington et.al, 1991).

A wide range of soil microorganisms including bacteria and fungi of plant rhizospheric soil releases organic acid.

There are almost forty one organic acids that came from microbial and fungal origin among which only a few are of the matter of interest from the view point of their commercial production viz citric, gluconic, fumaric, gallic, d-lactic, itaconic, Kojic and oxalic acids (Cochrane,1948).The organic acids of microbial origin are chiefly citric, gluconic, itaconic ,lactic, oxalic, fumaric , acetic and malic acids of which citric, acetic, malic, lactic and gluconic acids are of in industrial usage (Milson and Meers,1985;Moeller et.al.,2007)(CR=14).

Ren et.al. (2009); Christou et.al. (2006); Van Hees et.al, (2002) & Stevenson and Vance (1989) [CR=10] established that Oxalic, Citric and acetic acid can be produced by bacterial microorganisms at considerable level. Naraian and Kumari (2018) considered that major types of organic acids produced by the microorganisms are chiefly attributed to Citric , succinic , lactic , itaconic , lactobionic, gluconic, fumaric, propionic and acetic acids.

The purposes of these organic acids secretion by microbes are chiefly attributed to make various insoluble mineral elements of soil, being made available to the plants in their soluble form (Morgan et.al, 2005, Balogh – Brunstad et. al, 2008). Vidyashree et.al, (2018) studied the potentiality of organic acid production by micro organisms aiding in Zinc from rhizospheric soil as Zn^{2+} divalent ion of Zinc by releasing the cations through the action of organic acids excreted in the rhizospheric environment by the micro organisms that plays a pivotal mechanisms in Zinc biosolubilization (Vidyashree et. al 2018). Additionally, the anions can increase Zinc solubility through chelation of Zinc in order to convert them in their enhanced bio available form (Zn^{2+}) available to the crop plants (Jones and Darrah 1994) Similar microbial conversion of inorganic, insoluble phosphorous becomes also evident into their soluble bio available forms which make the insoluble forms of phosphorus readily available to the plants as a result of which, increased soil fertility and crop productivity in association with high social, economical and environmental benefits also gets achieved (Serna passo et.al,2017).

MATERIALS AND METHODS

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.

Enrichment of Soil Samples

Enrichment of soil samples from sampling location site was done with modified Biebl and Pfennig's medium (1981) through sequential incubation in dark and phototrophic condition at $32\pm 2\text{ C}^\circ$ anaerobic environment until it reach the O.D. around 0.5 at 600 nm. This enrichment was done following the standard protocol cited by (Madigan *et.al.*, 1984, Ponsano *et.al.*, 2002 & 2008). The growth obtained in this way was further enriched with modified RCVBNP medium without altering other factors and parameters except pH that was adjusted to 6.8 before it's autoclaving following general enrichment protocol as described by Madigan and Gest (1982).

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 ± 200 lux) at $32\pm 2\text{ C}^\circ$ (Ponsano,*et.al.*,2003) following a method as described by Archana *et.al.*, (2004).

Purification of Purple Non Sulfur Bacterium (PNSB)

It was done by repetitive streaking on the above medium and finally single bacterial colonies were picked up and maintained by sub culturing successively in modified RM2 medium (Sinha 1992).

Enrichment of Purple Non Sulfur Bacterium (PNSB)

Enrichment of Purple Non Sulfur Rhizobacteria was done following standard protocol of Winogradsky column method of enrichment for anoxygenic phototrophic bacteria (Yasa *et.al.*, 2006) and growth obtained by this way was further enriched with RCVBNP medium following general enrichment protocol of Madigan and Gest (1982).

Maintenance of Purple Non Sulfur Bacterium (PNSB)

Stock culture of Purple Non Sulfur Rhizobacteria were maintained on both agar stabs and broth cultures prepared separately with modified Biebl and Pfennig's agar medium (1981) and modified RM2 medium (Halder 2001, Sinha1992) preserved under 4 C° refrigeration after incubated in anaerobic condition. For long term storage, cryopreservation was done in liquid cultures with 30% glycerol supplementation in -270 C° refrigeration (Yurkov and Beatty, 1998).

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).

Preparation of Culture Supernatant from Bacterial Culture

The stabbed stock cultures were used as source of inoculums and further incubated on modified RM2 medium (Halder 2001, Sinha 1992) under anaerobic condition with continuous light illumination at 1400 ± 200 lux and $32 \pm 2^\circ\text{C}$ (Ponsano *et.al.*, 2002) temperature for 6-7 days of growth and this culture was preserved under 4°C . This 6-7 days old freshly prepared bacterial culture was used for 2-3 times subculture in order to get very freshly prepared live bacterial culture with active logarithmic growth for their further experimental usage. After the incubation period, freshly grown bacterial cells were thoroughly removed from the culture medium through centrifugation at 4°C under $5,500 \times g$ for 10 min (Patten and Glick 2002) by discarding the cell pellets prior to collect the bacterial supernatant which later on passed through the nitrocellulose membrane filter (0.5μ) to get the final supernatant fluid.

Screening of Organic Acid Production (*in vitro*)

Screening of the bacterial potential for organic acid production was done by a method as described by Cappuccino and Sherman (1992).

Briefly, 0.1 ml of inoculums, taken from 5 days old freshly grown bacterial culture was incubated in sterile nutrient agar medium supplemented with 5% Sucrose & 1% CaCO_3 under anoxygenic anaerobic condition with continuous illumination of 1400 ± 200 lux (Ponsano, *et.al.*, 2002) at room temperature for 48 hrs and then subjected to observation at a periodic time interval for detection of dissolved CaCO_3 zone for identification of the organic acid production ability by the bacterial Strain visible against white background due to CaCO_3 dissolution by the organic acid produced by the bacterium.

Formation of clear zone around the bacterial Colony due to dissolution of CaCO_3 by the organic acid produced by the bacterium established the bacterial potentiality for organic acid production.

Determination of Acid Unitage (AU) Value

The acid unitage (AU) value of the organic acid producing PNSB bacterial colonies were determined by a method as described by Shaikh and Quereshi, (2013).

The AU values were calculated by dividing the diameter values of clear zones by that of the bacterial colonies. AU values were determined by measuring the colony diameter (mm) and the diameter of the clear zones surrounding the colonies through subsequent division. The result was represented in tabular form.

Paper Chromatographic Study of Supernatant for Bacterial Organic Acid Production

The bacterial culture supernatant was concentrated prior to their spotting on chromatographic paper along with the organic acids used as standards.

The chromatographic chamber was presaturated with a solvent mixture containing n-butanol, acetic acid and water in 12:3:5 ratio (Sahoo and Gupta, 2017) in which the chromatogram was run for 24 hours, it was then air dried and sprayed with bromocresol green reagent. The *R_f* values of the yellow spots of the microbial organic acids, visible after developing on blue background was measured and compared with that of the organic acids, used as standards. Matching and comparing of two *R_f* values confirmed the identification of the organic acids of microbial origin present in supernatant.

RESULT

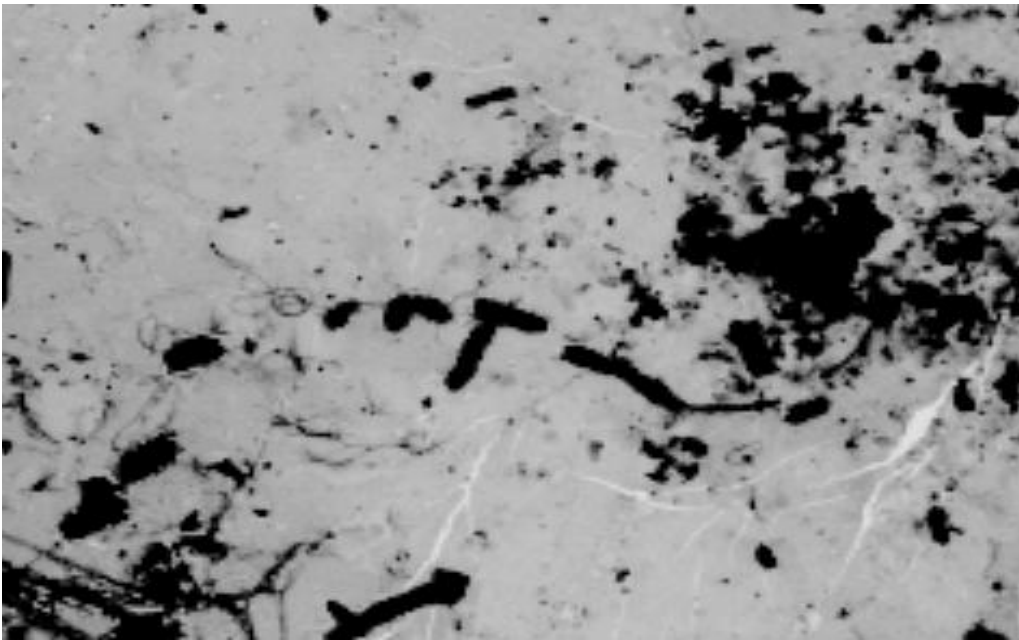


Fig: 1. SEM of *Rubrivivax gelatinosus* Strain RASN4

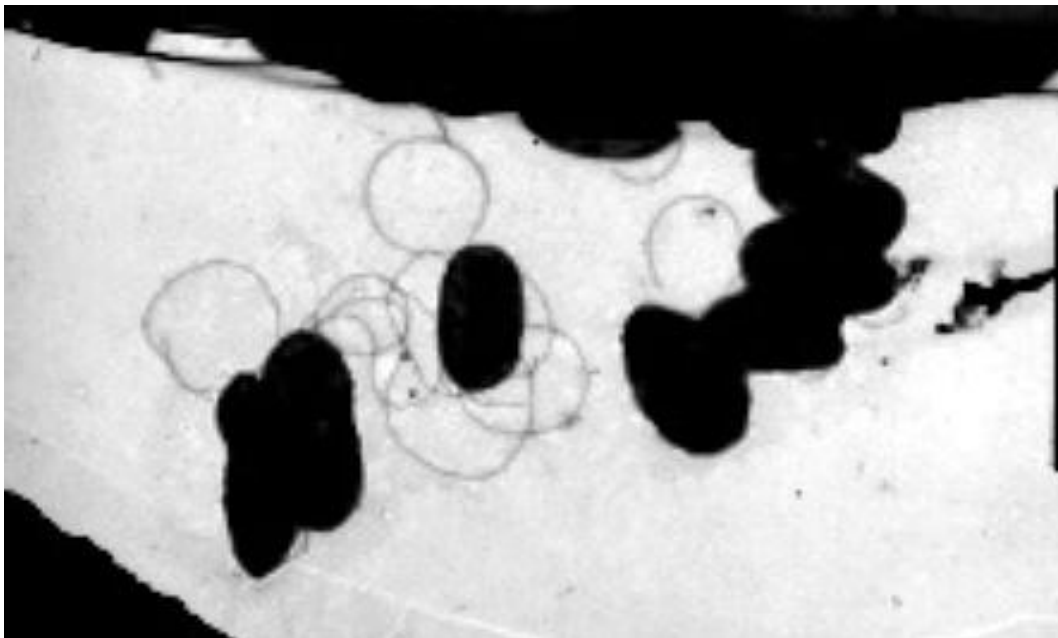


Fig: 2. TEM of *Rubrivivax gelatinosus* Strain RASN4

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

Sl. No.	Characteristics	Bacterial Isolates <i>Rubrivivax gelatinosus</i> (RASN4)
1.	Growth	Photoautotrophic
2.	Color of Anaerobic Culture	Pale Peach to Red
3.	Cell Shape	Curved Rod
4.	Size (Width × Length)	(0.3 - 0.7 × 1.3 – 2.1) μm
5.	Gram Staining	Gram negative
6.	Flagellation	Polar, Monotrichous
7.	Motility	+
8.	Formation of Sheaths	-
9.	Slime Production	+
10.	Growth Temperature Range (°C)	5 – 45°C
11.	Salt Tolerance Level	5%
12.	Nitrogen Fixation Ability	+
13.	Catalase Activity	+
14.	Urease Activity	-
15.	Oxidase Activity	+
16.	NO ₃ ⁻ Reduction Test	+
17.	Casein Hydrolysis	+
18.	Starch Hydrolysis	-
19.	Gelatine Liquefaction (Gelatinase)	+
20.	Hughe-Leiffson (O/F) Reaction Test	O/F
21.	IMViC Test Indole production Test Methyl Red Test Vogese Proskauer Test Citrate Utilization Test	+ - - +
22.	Bacteriochlorophyll	+
23.	Carotenoid	+
24.	Poly-Beta-Hydroxybutyrate (PBHB)	+
25.	Exo-Polysachharides (EPS)	+
26.	Growth Accelerators	Thiamine, Biotin

Sl. No.	Characteristics	Bacterial Isolates <i>Rubrivivax gelatinosus</i> (RASN4)
1.	Utilization of Carbon Source	
	Acetate	+
	Butyrate	ND
	Cytrate	+
	Formate	+
	Fumarate	+
	Glutamate	+
	Lactate	+
	L-Malate	+
	Propionate	+
	Pyruvate	+
	Succinate	+
	Tartate	+
	Arabinose	
	Cellbiose	+
	Fructose	-
	D-Galactose	+
	Glucose	ND
	Lactose	+
	Maltose	+
	Raffinose	-
	Rhamnose	-
	D-Ribose	-
Sucrose	ND	
Xylose	+	

	Sorbitol	+
	Mannitol	
	Glycerol	-
		-
		-

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

Table-2: Acid unitage (AU) values of isolated PNSB bacterium *Rubrivivax gelatinosus* (Strain RASN4) after five (5) days old incubated bacterial culture on modified RM2 medium.

Bacterial Culture	Zone Diameter (mm) of Bacterial Colony	Zone Diameter (mm) of Clear Halo	Acid Unitage Value (AU)
RASN4-1	0.57	1.69	2.93
RASN4-2	0.60	1.80	3.00
RASN4-3	0.58	1.72	2.96
RASN4-4	0.65	2.02	3.10
RASN4-5	0.57	1.64	2.87
RASN4-6	0.59	1.73	2.93
RASN4-7	0.63	1.86	2.95
Average	0.60	1.78	2.96
Min	0.57	1.64	2.87
Max	0.65	2.02	3.10
S.D.	0.031	0.128	0.072
S.E.	0.012	0.048	0.027

Graph showing acid unitage (AU) values of isolated PNSB bacterium *Rubrivivax gelatinosus* (Strain RASN4) after five (5) days old incubated bacterial culture on modified RM2 medium.

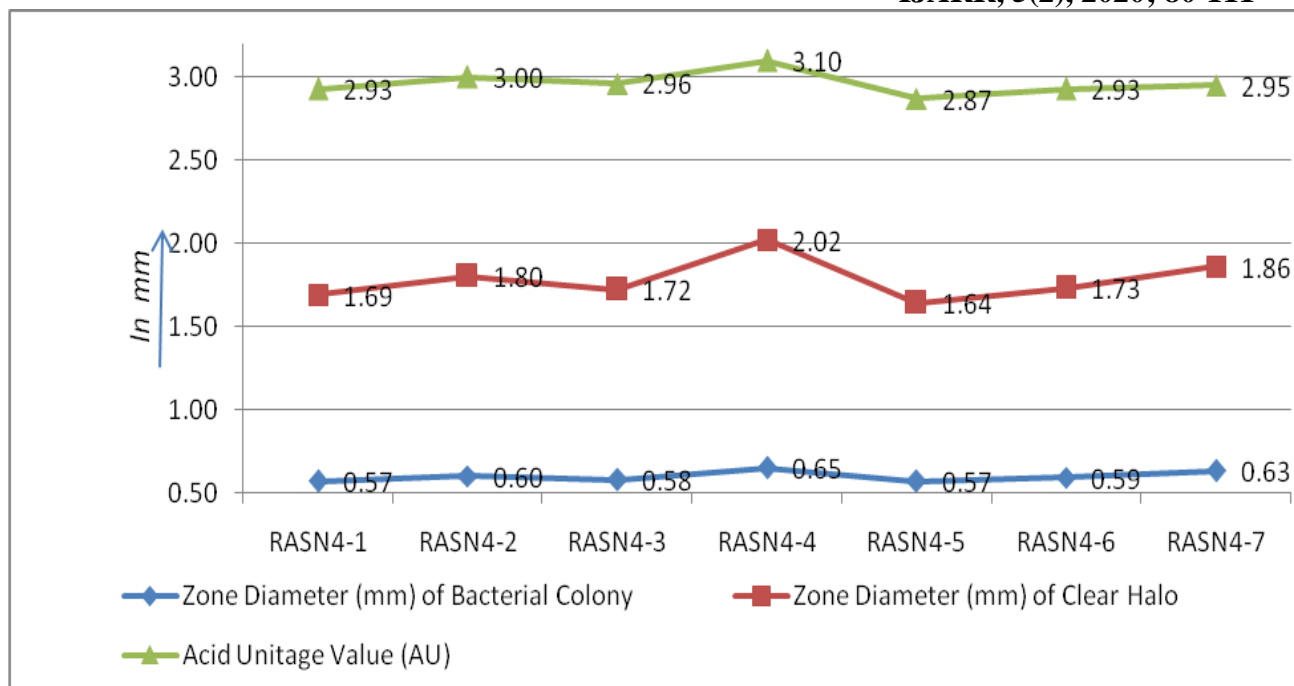
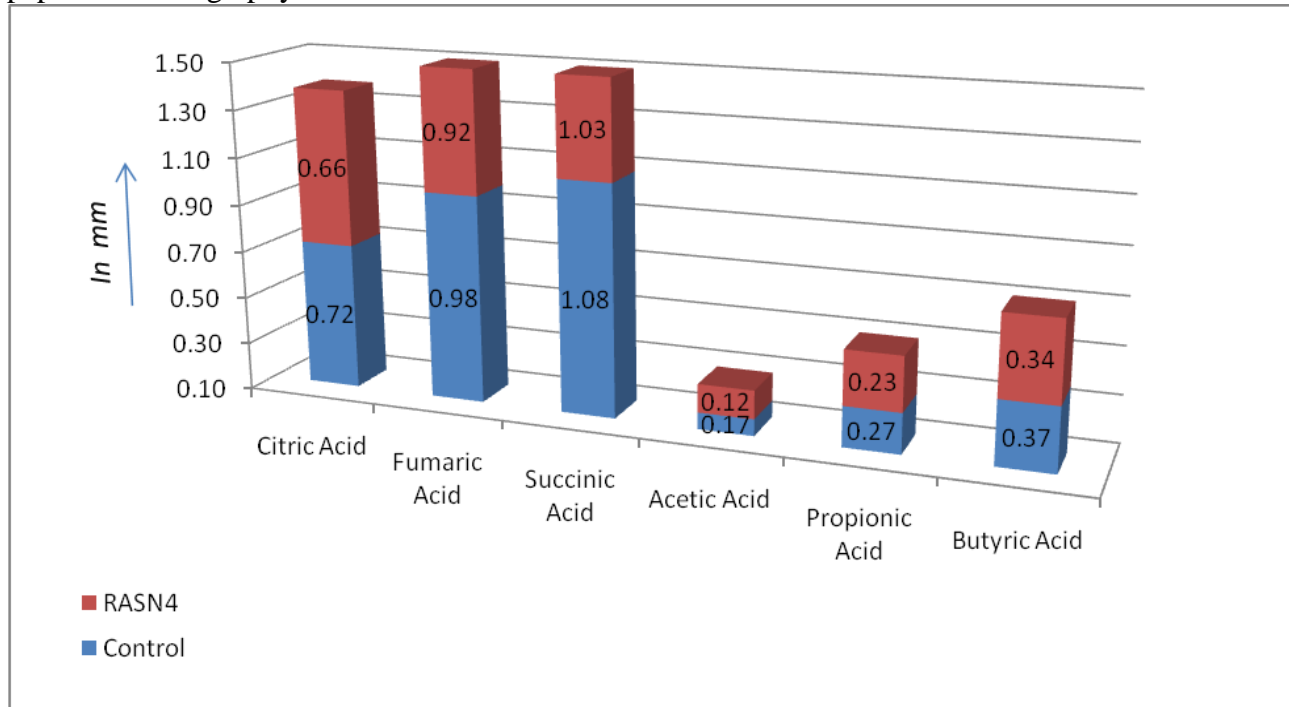


Table-3: Qualitative analysis of exogenous organic acids produced by isolated PNSB bacterium *Rubrivivax gelatinosus* (Strain RASN4) after five (5) days of incubation on modified RM2 medium in terms of their comparative *R_f* Values with known reference control on paper chromatography.

Exogenous acid in bacterial Medium	Control (<i>R_f</i> Values)	RASN4 (<i>R_f</i> Values)
Citric Acid	0.72	0.66
Fumaric Acid	0.98	0.92
Succinic Acid	1.08	1.03
Acetic Acid	0.17	0.12
Propionic Acid	0.27	0.23
Butyric Acid	0.37	0.34
Average	0.60	0.55
Min	0.17	0.12
Max	1.08	1.03
S.D.	0.384	0.377
S.E.	0.157	0.154

Bar diagram showing Qualitative analysis of exogenous organic acids produced by isolated PNSB bacterium *Rubrivivax gelatinosus* (Strain RASN4) after five (5) days of incubation on

modified RM2 medium in terms of their comparative *R_f* values with known reference control on paper chromatography.



RESULT AND DISCUSSION

The isolate *Rubrivivax gelatinosus* RASN4 strain was screened for qualitative detection of their *in vitro* organic acid production potentials as summarized in Table-2. Isolate *R. gelatinosus* RASN4 was proved to be strongly positive for *in vitro* organic acid production as indicated by the formation of clear zones around the bacterial Colonies (Table-2) followed by the determination of its acid unitage (AU) value.

This became further evident from the paper chromatographic study for the assessment of *in vitro* organic acid production potentials (Table-3) of isolated *Rubrivivax gelatinosus* strain RASN4.

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

Isolation, screening and Purification of effective PNSB isolate of *Rubrivivax gelatinosus* Strain (RASN4) was performed after being isolated from the rice rhizospheric soil of Ramnagar, located at Hooghly district of West Bengal, India. After then it was purified thoroughly by reparative streaming followed by their subsequent sub culturing, prior to their further characterization in order to identify them properly. All of the seven replicates of the isolated bacterial Strain RASN4 were grown up successfully in both of the nutrient agar (NA) (Atlas, R.M., 2010) and modified RM2 medium (Sinha 1992). Under the condition of bacterial growth as described by Pousano *et.al.* (2003) prior to their further characterization.

b) Physiochemical characterization of *R. gelatinosus* Strain RASN4

The isolated PNSB bacterial RASN4 Strain were first subjected to their normal microscopic study followed by their scanning and transmission electron microscopy (SEM & TEM) (fig.) revealing the shape of the individual bacterial cell as curved rod in nature with a size ranged from 0.3-0.7×1.3-2.15 μm (width x length). This study also revealed their flagellation pattern as polar and monotrichous in nature with positive sign of motility and slime production as became

evident through further study. The salt tolerance level of their growth was recorded at 5% level and growth of temperature was ranged between 5-45°C. Their further physiochemical characterization was done based on Burgey's manual (1986) and results were summarized in Table.

c) Identification of isolated PNSB *R. gelatinosus* strain RASN4

The isolated PNSB RASN4 Strain was further subjected to their confirmed Identification up to genus and species level through their metabolic fingerprinting based BIOLOG™ Identification system, subsequently followed by their 16S rRNA analysis. From the results, obtained from the both ends (not included here), the rice rhizospheric PNSB RASN4 Strain was confirmly identified as *Rubrivivax gelatinosus* of the family Comamonadaceae that belongs to the order Burkholderelias of beta-proteobacteria group.

d) *In vitro* screening for exogenous production potential of organic acids by isolated PNSB strain RASN4

The present study confirmed that potential Capability of *in vitro* organic acid production by isolated PNSB bacterium *Rubrivivax gelatinosus* RASN4 in order to exploit this quality for bioformulation of a potent microbial bio fertilizer with adequate potentiality of geomineral biosolubilization. Evaluation of production potential of exogenous organic acids (*in vitro*) through qualitative screening established the strong positive indication of *in vitro* organic acids production potentials as summarized in Table: Qualitative analysis of exogenous organic acids produced by RASN4 Strain was performed after 5 days of incubation on modified RM2 medium (without altering the other growing conditions) in terms of their comparative *Rf* values with reference control of known specific organic acids using as control on paper chromatography.

The *Rf* values obtained from culture supernatant of isolated RASN4 Strain on paper chromatography were found as 0.66,0.92,1.03,0.12,0.23 & 0.34 respectively which were compared with their specific reference Control, prepared using different known specific organic acids that coincided with Citric(0.72),fumeric(0.98),succinic(1.08),acetic(0.17),propionic(0.27),butyric acid (0.37) respectively when compared to the standard known organic acids used as reference Control.

This became further evident from the determination of acid unitage (AU) values of the isolated of PNSB Strain RASN4 as represented in Table: ,after 5 days old incubation of isolated bacterial Culture on modified RM2 medium again without altering the other growth conditions.

Acid unitage Values (AU) were determined by measuring the diameter (mm) of isolated bacterial Strain Colony and that of halo zones formed around them substantiating the existence of exosecretion of organic acids from isolated bacterial origin.

The acid unitage (AU) Value, diameter (mm) of isolated bacterial colony growth and diameter (mm) of halo zones were represented in Table: in which all of the seven replicates of isolated bacterial RASN4 Strain showed promising performances in Terms of their significant organic acid a production. The highest(AU) value was shown by the 4 the replicates with the value of 3.11 where as the lowest value observed as in case of the 5th replicate with a value of 2.88. The average acid unitage (AU) value for isolated PNSB *Rubrivivax gelatinosus* Strain RASN4 was 2.96, confirming their potentially of bacterial organic acids production capability.

Organic acids are chiefly those organic Carboxylic Compounds that comprise of long chain of Carbons with attached functional Carboxylic groups, having acidic properties in association with

the same (Shaikh and Qurshi, 2013). Many bacteria including PGPR are capable of producing exogenous organic acids along with their other PGPR potential traits in order to play their prime role in promoting of plant growth and development with enhancement of crop productivity, achieved through the microbial biosolubilization of various insoluble, inorganic macro and micro trace metal elements essential for plants by converting them into their solubilize bio available forms for the plants (Vidyashree et.al.,2013).

Although many bacterial strains including plant growth promoting rhizobacteria (PGPR) are capable of producing *in vitro* exogenous organic acids, purple non sulfur bacterial members too are not exceptional in this context. But their *in vitro* organic acid production is chiefly constituted of indole-3-acetic acid (IAA) and 5- aminolevulinic acid (ALA) production except in some cases where only a few other acids like acetic, oxalic, gluconic, lactobionic, butyric acid etc. are exogenously synthesized from their microbial origin either separately or in a combination. As the potentiality for *in vitro* production of IAA and ALA has already been established (not included in present study) for this isolated rice rhizospheric PNSB Strain *Rubrivivax gelatinosus* RASN4; so, this present work is concentrated on the capability of *in vitro* exogenous production of organic acids other than these two (IAA and ALA) by this said RASN4 Strain. *In vitro* exogenous production of Indole-3-acetic acid (IAA) by various PNSB bacterial strains were established confirmedly by several researchers.

Li *et.al*, (2018) studied that PNSB bacterial strain *Burkholderia multivorans* has the potentiality for *in vitro* exogenous IAA production. Wang *et.al*, (2016) found that *Burkholderia helia* is also capable of producing *in vitro* IAA in bacterial medium. Rajyalaxmi and Girisham (2015) studied the IAA production potential of two anoxygenic phototrophic purple bacteria viz. *Allochromatium sp.* and *Rhodobacter sp.* in presence and absence of inducer and found that *Allochromatium sp.* can produce highest amount of their *in vitro* IAA production (165 µg/ml) and other two *Rhodobacter sp.* strains can show their capability of *in vitro* IAA production (120 µg & 140 µg) on 8th and 10th day of the incubation respectively. Mohammed *et.al*, (2014) and Mujahid *et.al*, (2011) showed that *Rubrivivax benzoatilyticus* is capable of producing IAA and related indole derivatives. Prasuna *et.al*, (2012) also established that phototrophic PNSB bacterium *Rubrivivax benzoatilyticus* having the potentiality for *in vitro* phenyl acetic acid production through L-phenyl alanine catabolism.

Kumavath *et.al*, (2010) confirmed the occurrence of indole-3-pyruvic acid pathway present in PNSB strain *Rubrivivax benzoatilyticus*. Munjam *et.al*, (2002) established that potentiality of *in vitro* IAA production by two anoxygenic phototrophic bacteria *Rhodocyclus gelatinosus* (later known as *Rubrivivax gelatinosus*) and *Rhodobacter Sphaeroides*.

Microbial production of 5-aminolevulinic acid was also established by Liu *et.al*, (2014). Previously Andersen *et.al*, (1983) worked on mechanism of 5-aminolevulinate synthesis in purple, green and blue-green bacteria. Sasaki *et.al*, (1987) showed the capability of *in vitro* 5-aminolevulinic acid production by photosynthetic bacteria. Sasaki *et.al*, (1991) confirmed the *in vitro* enhanced production of 5-aminolevulinic acid by photoheterotrophic PNSB culture of *Rhodobacter Sphaeroides*. Nishikawa *et.al*, (1999) established the potentiality of *in vitro* 5-aminolevulinic acid accumulation by PNSB bacterial strain *Rhodobacter Sphaeroides* mutants under dark aerobic condition.

Chaikritsadakarn *et.al*, (2006) established PNSB strain *Rhodobacter Capsulatus* having the potentiality of *in vitro* 5-aminolevulinic acid production. Saikour *et.al*, (2009) confirmed the production of extra cellular 5- aminolevulinic acid by PNSB bacterium *Rhodospseudomonas palustris*. Mario *et.al*, (2017) established the potentiality of photosynthetic PNSB bacterium *Rhodospseudomonas palustris* for *in vitro* 5-aminolevulinic acid production. Nunkaew *et.al*,

(2018, 2014) confirmed enhanced 5-aminolevulinic acid production by PNSB bacterial strain *Rhodospirillum rubrum* by using wood vinegar as ingredient. Potentiality of 5-aminolevulinic acid production by photosynthetic PNSB bacterium *Rubrivivax* spp. was also been established by various researchers. Sattayasamitsathit and Prasertsan (2013) characterized a newly isolated *Rubrivivax benzoatilyticus* with potentiality of 5-aminolevulinic acid production. Lim *et.al.*, (1993) optimized intact cell system of *Rhodocyclus gelatinosus* (later known as *Rubrivivax gelatinosus* for *in vitro* delta aminolevulinic acid production).

Apart from the other organic acids, a very few have been detected through their *in vitro* production study by PNSB bacterial strains as confirmed by some of the workers.

Li *et.al.*, (2018) postulated that *in vitro* organic acid secretion by the anoxygenic PNSB bacterium *Burkholderia multivorans*, isolated from poplar root rhizospheric soil was directly contributed by both *in vitro* production of gluconic acid and IAA of microbial origin.

Wang and Liu (2014) found that *in vitro* production of (R)-3-hydroxy butyric acid is potentially possible by PNSB bacterial strain *Burkholderia cepacia*.

Nakata and He (2011, 2010) established that PNSB bacterial strain named *Burkholderia glumae* can synthesise oxalic acid in their *in vitro* production.

So, after obtaining the result from the both end followed by their comparing, it can be postulated that the rice rhizospheric PNSB bacterium *Rubrivivax gelatinosus* (RASN4) has the significant potentiality for showing promising performance for microbial organic acid exoproduction along with its other traditional PGPR traits. This is also the first time report of organic acid exoproduction by PNSB bacterium strain *Rubrivivax gelatinosus* (RASN4) as became evident through qualitative screening of the present study that can be exploited to bioformulate a potential microbial biofertilizer for the benefit of the agriculture and mankind.

CONCLUSION

In conclusion, it may be summarized that the purple non sulfur bacterial Strain (RASN4) isolated from the rice field of Ramnagar of Hooghly district of West Bengal, India (Latitude 22.82N; Longitude 87.80E) which has been identified as *Rubrivivax gelatinosus* is capable of producing exogenous organic acids as found through their qualitative screening and has also later been confirmed through determination of their acid unitage (AU) value,

Organic acids, exogenous from microbial origin plays a key role in plant growth and development along with the enhancement of their crop productivity through the microbial biosolubilization of various inorganic macro and micro trace elements metals by converting them from their inorganic insoluble unavailable to solubilize bioavailable form for the plants (Vidyashree *et.al.*2018) with special reference to Zinc (Zn) (Vidyashree *et.al.*,2018; Jones and Darrah,1994) and phosphorous(P) biosolubilization (Saikia,2018; Serna passo *et.al.*,2017; Mardad *et.al.*,2013; Sharma *et.al.*2013; Oburger *et.al.*,2011; Scervino *et.al.*2010; Vyas and Gulati 2009, Malboobi *et.al.*2009, Chen *et.al.*2006; Sahin *et.al.*2004; Srivastava *et.al.*,2003; Kang *et.al.*,2002, Gilroy and Jones 2002; Strobel, 2001; Whitelaw 2000).

Such potentiality of exogenous organic acid production by PNSB isolated Strain RASN4 might have been exploited in near future for bio formulation of an efficient microbial biofertilizer bioinoculant with potentiality of giomineral bio solubilisation in order to make them more biologically available to the agricultural crop and vegetable plants contributing directly to their plant growth, vigor, development and production yield as a whole in terms of agricultural crop productivity in Indian sub-continent.

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