International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Biocontrol Efficiency of *Pseudomonas* species against the Algal Blooms in the Eutrophicated pond water

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Abstract

This study was done on eutrophicated water samples with *Pseudomonas* species. The aim of this study was to isolate and identify the antagonistic bacteria and to check its efficiency as algicides and its host range. In this study two types of *Pseudomonas: Pseudomonas aeruginosa* and *P. putita* were applied. As a result their efficacy was noted and compared. It was clearly noted that *P. putida* shows quicker response than *P. aeruginosa*. From the results *P. putida* has high algicidal efficiency; it is able to inhibit the algal growth. Hence the biocontrol would be the best opinion in this case since applying chemicals or investing more expensive treatments. The advantage of biocontrol agent is specific to agents, without genetic modification and has control over algal growth. The GCMS study clearly showed totally 15 compounds were identified such as 7,9-Di-tert-butyl-1-oxospira (4,5)dec-6 shows highest peak compound followed by other peak fractions of the bioactive compounds were observed such as 2,4- Imidazolidinedione,5-[3,4-bis],10 methyl8-tetradecan-1-ol acetate,4-phosphatricyclo [6.1.1.0(2,6)] dec-2(6). Hence the present study clearly showed that the *P. putida* bacterium has immensed algicidal efficiency than *P. aeruginosa* because it could be inhibit or resist the algal growth (minimized the algal bloom) on eutrophicated pond.

Keywords: Biocontrol, *Pseudomonas putida, P. aeruginosa*, Algal bloom.

Introduction

Eutrophication – the enrichment of water bodies with plant nutrients, typically nitrogen and phosphorus and the subsequent effects on water quality as biological structure and function. Excessive algal and rooted plant growth, degraded water quality, extensive deoxygenating of the bottom water layers and increased fish biomass accompanied by decreased harvest quality are some features of this process. . One example is the "bloom" or great increase of phytoplankton in a water body as a response to increased levels of nutrients. Negative environmental effects include hypoxia, the depletion of oxygen in the water, which causes a reduction in specific fish and other animals. (Walter and Jeffrey, 1996).

Eutrophication is a common phenomenon in coastal waters. In contrast to freshwater systems, nitrogen is more commonly the key limiting nutrient of marine waters; thus, nitrogen levels have greater importance to understanding eutrophication problems in salt water. Estuaries tend to be naturally eutrophic because land-derived nutrients are concentrated where run-off enters a confined channel. Upwelling in coastal systems also promotes increased productivity by conveying deep, nutrient-rich waters to the surface, where the nutrients can be assimilated by algae.

Toxicity data provided by Oliveira - Filho *et al.*, (2004) confirm that planktonic crustaceae and algae are extremely susceptible to increases in free copper levels in water bodies. Since phytoplanktonic and zooplanktonic organisms form the basis of aquatic food webs, increased levels of bioavailable copper can dramatically affect freshwater ecosystems. Herbicides

such as diuron, simazine, atrazine and they block the electron flow in photosystem II and are known to inhibit the growth of algae especially *Microcystis*. But the xenobiotic nature of these herbicides seems to prevent its further use as algicides.

An increasing number of laboratory studies and field application have shown that plant mulch with allelopathic activity could also be used to control aquatic weeds and algal growth. Barley straw has long been known for its algal control property in fresh water ecosystems (Newman and Barrett, 1993; Everall and Lees, 1997; Caffey and Monahan, 1999; Terlizzi *et al.,* 2002; Brownlee *et al.,* 2003). It was extensively used in the British Isles lakes, potable water reservoirs, canals and streams and was proved successful (Welch *et al.,* 1990). But the results were varying in North America and the reasons for this variation remain unclear (Boylan & Morris, 2003). The factors that must be properly addressed for the success of barley straw for freshwater algal control include adequate straw dosage, starting treatment well in advance of bloom development, proper positioning of the straw in the body of water, adequate aeration of the straw and adequate water circulation.

Several studies have demonstrated that extracellular substances such as hydroxylamine (Berger *et al.,* 1979) ; phenazines (Dakhama *et al.,* 1993) ; aminophenol (Yamamoto *et al.,* 1998); rhamnolipids (Wang *et al.,* 2005) ; protease (Lee *et al.,* 2000) ; bacillamide (Jeong *et al.,* 2003), Surfactin (Ahn *et al.,* 2003) and sophorolipid (Sun *et al.,* 2004) are algicidal in nature.

Short-term strategy to eliminate or reduce algal bloom generally involves chemical or biological in situ treatment. The usage of chemicals, most probably copper salts, was the conventional way of algal elimination from fresh water bodies (Hawkins & Griffths,1987 ; Meador *et al.,*1998 ; Han *et al.,* 2001 ; Oliveira-Filho *et al.,*2004). Copper treatments can result in potentially high levels of Cu in the surface waters and sediments (Hullebusch *et al.,*2003). Another major drawback of chemical treatments is that they are non selective and adversely affect a variety of non-target aquatic species (Meepagala *et al.,* 2005).
Eutrophication poses a problem not only to ecosystems, but to humans as well. Reducing eutrophication should be a key concern when considering future policy and a sustainable solution for everyone, including farmers and ranchers, seems feasible. While eutrophication does pose problems, humans should be aware that natural runoff (which causes algal blooms in the wild) is common in ecosystems.

Commercial application of algicides is, however, limited to copper compounds especially copper sulphate as in products such as clearigate and cutrine-plus. Therefore, the aim of this investigation is to find out an environmentally safe algicide of microbial origin.

Materials and Methods

Sample Collection

The water samples were collected from different waterbodies. The collected sample were placed in sterile bottles and brought to laboratory for analysis.

Physical Characterization of Water

The water samples were analyzed for physical parameters like colour, odour and turbidity before treatment.

Chemical Characterization of Water and Detection of Planktonic Diversity

The water sample were analyzed for chemical parameters like p^H, total nitrogen content, total calcium content, total phosphorus content, total chlorine content,total sulphur content, total magnesium content, BOD, COD, Dissolved Oxygen (DO) etc. followed by the standard procedures of Rajan, (2013). The water sample was examined under microscope to find out the different planktons in water bodies based on their morphological character.

Enumeration, Isolation and Identification Of Algae From Water Sample

The water samples were serially diluted and plated using different medias such as cyanophycean agar and BG11 agar. Incubate for one week at sunlight. The developed colonies were counted on plates. The nature and appearance of colony were noted. The isolated cyanobacteria were identified on basis of classification of scheme published in Bergey's manual.

Isolation of *Pseudomonas* sp. from the soil

Petroleum contaminated soil samples were collected from the places of Karamana. Serially diluted the soil samples and plated on cetrimide agar or *Pseudomonas* isolation agar.

Identification of Bacterial Isolate

Generic level identification of the isolates was carried out following Oliver (1982) and demarcated into species following Alsina and Blanch (1994). The tests carried out were Gram staining, Motility test, IMViC tests, carbohydrate fermentation, TSI test, catalase test and nitrate reduction test.(Bergey's Manual).

Detection of Algicidal Activity

Bacterial inoculation flask method

Take 100 ml of water samples in a flask. Add 5ml of bacterial culture to one flask and labelled it as test and other kept as control. Observe each day for visual change in water samples.

Well diffusion method

Prepare the lawn culture of cyanobacteria on cyanophacean agar. Cut 3 wells at definite space on agar plate and add bacterial culture to the wells. Incubate it overnight. Observe it for zone formation from the lawn cult.

Bacterial Cell Density and Algicidal Efficiency-Co-Culture Assay

The algae and bacterial isolate was grown in algal medium in flasks and it was incubated at room temperature under the light intensity. The cell density of the algal species was determined on alternate days using a colorimeter.

Extractability of Algicidal Metabolite

Cultures of antagonistic bacteria, *Pseudomonas* sp. (3-5 days old) were centrifuged at 10,000 g at 4^0 C for 20 minutes. The supernatant was filtered through a series of filters including glass microfiber filter (GF/C, Whatman), cellulose acetate membrane (0.45 µm) and PVDF membrane (0.22 µm) and is used for further analysis. The filtrate was extracted with different

solvents such as chloroform, petroleum ether, hexane, methanol, ethyl acetate and a mixture of Chloroform: Methanol (2:1) to recover algicidal compound. 20 µl of the solvent fraction was impregnated on sterile filter paper disc and allowed to dry for 30 min. Control discs were kept with solvents alone. The discs were placed on algal lawn of *M. aeruginosa*.

Results and Discussion

Physical and Chemical Characterization

Physical properties like colour, odour and turbidity were determined. Based on the p^H analysis of water sample on before and after treatment, it was noted that the p^H changes from acidic to alkaline condition. It is due to the enzymatic release of *Pseudomonas putida* for the lysis of *Mycrocystis.* Slight p^H variation cannot affect aquatic microflora. Smith *et al* (2008) documented biodegradation of cyanotoxin only occur in water supplies which had a history of *G. raclborskii* blooms.

Table:1- Physical characteristics of water sample

Sample	Colour	Odour	Turbidity
S1	Green	Pungent	Turbid
S2	Pale green	Fowl smell	Turbid
S3	Green	Pungent	Turbid
S4	Slight green	Fowl smell	Turbid
S5	Green	Pungent	Turbid

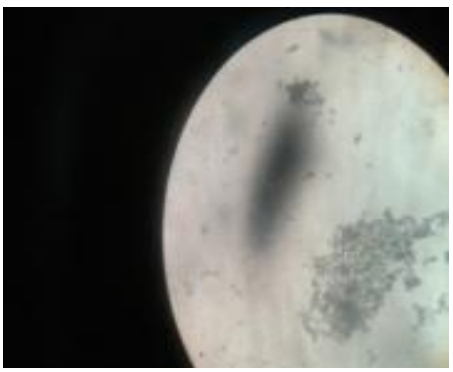
Chemical characteristics like nitrogen, chlorine, phosphorous, sulphur, calcium, BOD, COD and DO were In this study, on post water analysis, it was noted that the content of phosphorous get reduced and diminished. The phosphorous content of S_1, S_2, S_3 are 0.002, 0.001, 0.001 respectively before inoculation. After inoculation of *Pseudomonas putida*, the phosphorous content of S_1, S_2, S_3 are disappeared. This indicates that the *Pseudomonas putida* is capable of solubilizing phosphorous. The presence of water constituents may also influence biodegradation of organic compounds as metals have been shown to inhibit biodegradation of aromatic hydrocarbons (Amor *et al*, 2001).

Table: 2- Chemical characteristics of water sample, **A**: Before treatment. **B**: After treatment of eutrophic pond

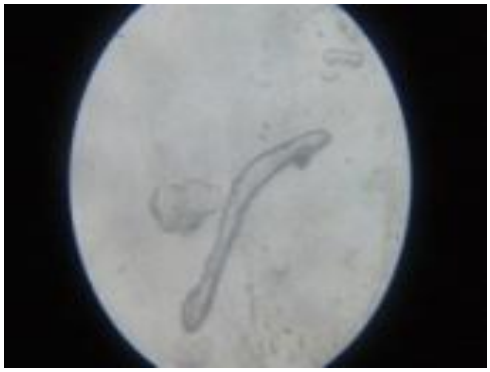
(A) PRE ANALYSIS					
Chemical constituents	**Sample**				
	S_1	**S_2**	**S_3**	**S_4**	**S_5**
pH	6	5	6	6	5
Nitrogen(ppm)	560	600	750	725	650
Chlorine (ppm)	225	280	260	230	265
Phosphate (ppm)	0.022	0.002	0.001	-	-
Sulphate (ppm)	600	650	800	625	750
Calcium (ppm)	210	205	225	300	-
DO (mg/l)	2	3	1	3	2
BOD (mg/l)	10	12	11.5	12	10
COD (mg/l)	16	14	12	14	13
B) POST WATER ANALYSIS					
pH	8	8	9	8	8
Nitrogen (ppm)	310	325	410	335	350
Chlorine (ppm)	200	210	206	190	215
Phosphate (ppm)	-	-	-	-	-
Sulphate (ppm)	350	400	650	310	325
Calcium (ppm)	125	101	150	115	-
DO (mg/l)	3	4	2	4	3
BOD (mg/l)	8	9	10	9	8
COD (mg/l)	11	11.5	10	11	10.5

Fig:- 1 Planktonic Diversity from the Eutrophicated Pond

Different types of planktons were identified under microscope.

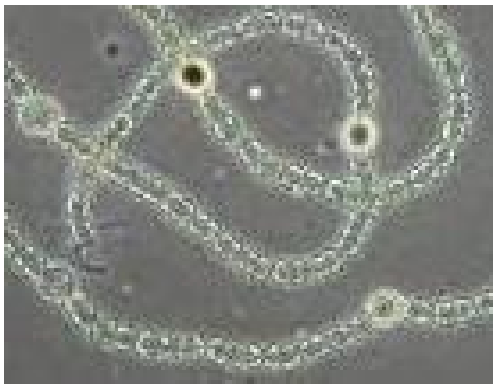


Microcystis



Oscillatoria

Euglena



Nostoc

Isolation of *Pseudomonas* sp. from soil

Fig:-2 Appearance of *Pseudomonas* growth on cetrimide agar (A) appearance of algal lawn growth on media (B)

Biochemical Characterization

The isolated strains were characterized. The characterization was based on microscopic and biochemical test results in confirmation with Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Table:3-Biochemical characterization of bacterial isolates

Property	IC_1 (*P. putida*)	IC_2 (*P. aeruginosa*)
Gram staining	Gram negative rods	Gram negative rods
Motility	Motile	Motile
Indole test	Negative	Negative
Methyl red test	Negative	Negative
Voges – Proskaeur test	Negative	Positive
Citrate utilization test	Positive	Positive
Carbohydrate fermentation test	Positive	Negative
Catalase test	Positive	Negative
Nitrite test	Negative	Positive
TSI test	A/K no gas no H_2S	A/K with gas

IC_1 – Isolated colony 1 IC_2 – Isolated colony 2

Detection of Algicidal Activity

Bacterial inoculation -Turbidity method

The test water sample changes from muddy to clear within 7 days after inoculating *P. putida* to the water sample. It indicates the clear evidence of algicidal activity of *P. putida* on eutrophicated water sample. The results obtained on direct inoculation method and well diffusion method gives a clear evident that *P. putida* can be used as algicide. Bio-control with indigenous bacteria does not pose a health threat and are an alternative solution to avoid build up of noxious cyanobacterial blooms. Based on cell density ratio studies, the initial OD value is very high due to increase in number of algal cells. On adding *P. putida*, the OD value get fluctuates and reduced. It is due to algal lysis. Thus the *Mycrocystis* loads get reduced. Manage *et al* identified three isolates, *Arthrobacter* sp., *Rhodococcus* sp., and *Brevibacterium* sp. as having the capability to degrade two microcystin variants.

Fig:-3 Appearance of water sample after inoculation:

A – Control **B – test**

Fig:-4 The algicidal activity of *Pseudomonas* by **Kirby Bauer method Well diffusion**
The algicidal activity was clearly indicated by clear zone of inhibition around algal growth.

Appearance of clear zone of *Pseudomonas* on algal lawn

Bacterial Cell Density and Algicidal Efficiency Co-Culture Assay

As the number of bacterial cell increases, the algicidal effect also increases. The cell density of *M. aeruginosa* declined at high concentration of bacterial inoculum when compared to control. The difference in algal cell count between treated densities of microbial inoculum. Study on the bacterial cell density on algicidal effectiveness depicts the low requirement of bacteria as inoculum. In order to check whether bacteria is directing attacking algae or any other secondary metabolites of algicidal nature are acting, bacterial filtrate was extracted with organic solvents such as chloroform, methanol, petroleum ether, ethyl acetate, hexane and a mixture of chloroform : methanol. Cell free filtrate of *Pseudomonas* extracts in chloroform, petroleum ether and chloroform: methanol mixture exhibited algicidal activity. The effect was more pronounced in chloroform extract. Klitzke *et al*. (2010) showed that in sediments, the presence of aquatic dissolved organic matter yield higher presence of cyanotoxin due to some sort of substrate specificity.

Table:4- Analysis of bacterial cell density ratio (in OD) of microbial inoculum on eutrophicated pond water sample

Sample	Optical density (nm)					
	Before inoculation	After inoculation				
		Days				
		1	2	3	4	5
I	0.62	0.88	0.97	1.05	0.78	0.55
II	0.68	0.90	0.99	1.10	0.85	0.62
III	0.50	0.65	0.87	0.99	0.68	0.45
IV	0.49	0.62	0.83	1	0.53	0.40
V	0.65	0.78	0.92	1.02	0.66	0.43

Extractability of Algicidal Metabolite

On extracting cell-free filtrate of *Pseudomonas putida* with five organic solvents and one mixture, algicidal activity was expressed in chloroform, chloroform: methanol and analysis petroleum ether. There was no activity in ethyl acetate, hexane and methanol fractions assessed by disc diffusion assay in *M. aeruginosa*. The chloroform extract produced the most distinct and largest clear zone. Algicidal activity was observed after 24 hrs. Host range of chloroform extract was studied in *Mycrocystis aeruginosa*. Dakhama *et al.*, (1993) reported that *Pseudomonas aeruginosa* strongly inhibited the growth of green microalgae and cyanobacteria. Green algal species is reported to induce polysaccharide production to protect it against oxidative stress caused by toxins (Mohamed, 2008). Nature of the cell wall and defensive strategy such as induced polysaccharide production may be the reason for less inhibitory effect on green algal cells.

Appearance of clear zone of cell free filtrates with organic solvents

GC – MS analysis

In this study, the different compound on blue green algae was identified on GC-MS analysis. Totally 15 compounds were identified and it is presented in table-5. Initially, 7,9-Di-tert-butyl-1-oxospira(4,5)dec-6 shows highest peak compound followed by other peak fractions of the bioactive compounds were observed such as 2,4-Imidazolidinedione,5-[3,4-bis],10 methyl8-tetradecan-1-ol acetate,4-phosphatricyclo [6.1.1.0(2,6)] dec-2(6). Moreover, the current result showed the both compounds named as 1,1-Cyclobutanedicarboxamide,2 phenyl and sucrose were lowest level or least peak present in the extracted algae. While the identified algicidal metabolites possessed many biological benefits especially eutrophication oriented compounds. Hence the GC-MS chromatogram depicted secondary metabolites were several biological activities relevant to this research.

Fig:1-GC-MS Chromatogram plot, Extracted algicidal metabolite

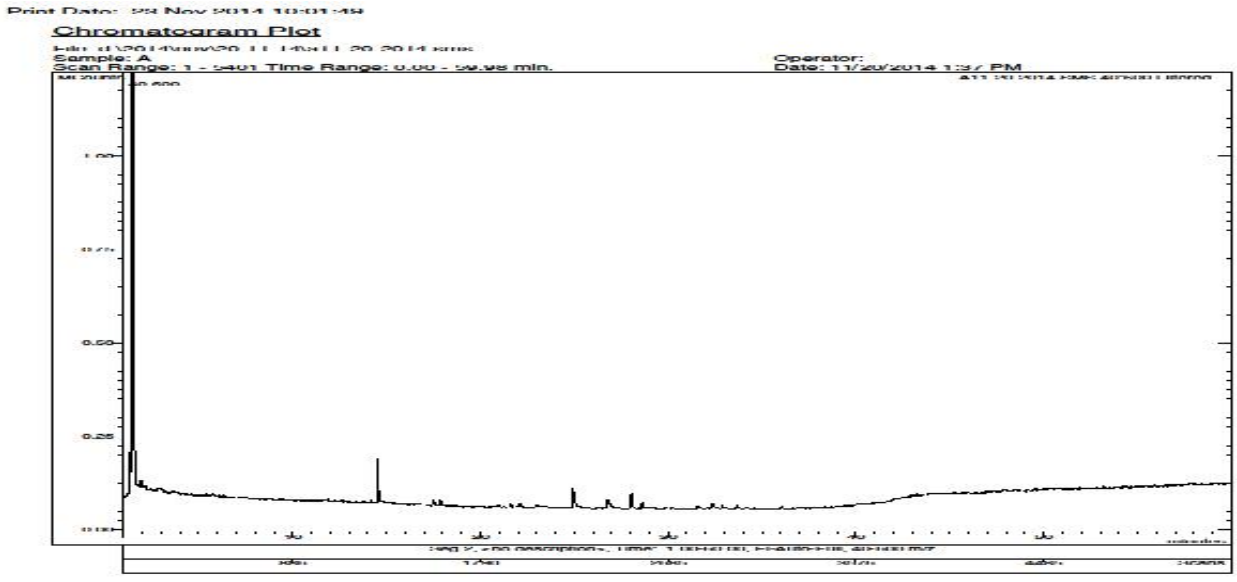


Table: 5- Illustration of biocompound analytes from the extracted algicidal metabolites by GC-MS analysis

Sl. No.	Compound name	Quan Ions
1	Sucrose	56.8
2	2,4-Imidazolilidinedione 5-[3,4bis	191.0
3	Tricyclo [5.4.30(1,8) tetradecan-6-one	148.9
4	4 Phosphatricyclo [6.1.1.0(2,6)] dec – 2(6)	150.8
5	1 Benzoxirene, 5a-[3-oxo-1-butenyl] perhy	122.9
6	4a,7a-Epoxy-5H-Cyclopenta[a] cyclopropa	135.0
7	9-Desoxo-9-x-acetoxy-3-desoxy-7.8.12-tri	124.0
8	10 methyl 8-tetradecen-1-ol acetate	151.9
9	Di-n-octylphthalate	148.9
10	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6	205.0
11	Pentadecanoic acid,13 methyl	142.8
12	1,1-Cyclobutanedicarboxamide,2 phenyl	56.8
13	2,4- Imidazolilidinedione 5-[3,4-bis(trim)]	142.9
14	Rhodopin	121
15	Oleic acid eicosyl ester	56.9

Conclusion

This study investigates that the use of a biocontrol agent to control algal bloom. For the study, *Pseudomonas* sp. was isolated and applied on eutrophicated water sample. Apart from the current results obtained it was found that *P. putida* has high algicidal efficiency than *P. aeruginosa*, it is able to inhibit algal growth. Consequently it leads to a finding that biocontrol agent is an efficient solution to avoid build up of noxious cyanobacterial blooms. Results were confirmed that the algicidal effect of *P. putida* on cyanobacterial bloom. In conclusion, *P. putida* isolated in the present study may provide a better strategy to reduce or control cyanobacterial algal bloom. Apart from the GCMS study clearly showed totally 15 compounds were identified such as 7,9-Di-tert-butyl-1-oxospira(4,5)dec-6 shows highest peak compound followed by other peak fractions of the bioactive compounds were observed such as 2,4-Imidazolidinedione,5-[3,4-bis],10 methyl8-tetradecan-1-ol acetate,4-phosphatricyclo [6.1.1.0(2,6)] dec-2(6). Further work is recommended to determine its application in lakes and reservoirs for water quality management. Biocontrol would be the best option in this case since applying chemicals or investing more expensive treatments. The advantage of biocontrol agent is specific to agents, without genetic modification and effect on algal bloom control.

Acknowledgments

This work would not have been possible without the assistance of the Mrs. Reena. T, Mr. Suresh, Mrs.Vijila Helen Mary and Mrs**.** K. Kochu Therasia and their diligent searching and patience are greatly appreciated. The assistance and patience of Mrs.Reena.T are gratefully acknowledged.

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