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Isolation and Characterization of Indigenous Bacterial Strains from Constructed Wetland with Biofertilizer Potential

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KEYWORDS

Biofertilizers, Phosphate Solubilizing Bacteria, Nitrogen Fixing Bacteria, Soil Fertility, constructed wetland, Nutrient Management, Rhizosphere

ABSTRACT:

Recent advancements in agricultural practices have significantly increased the use of chemical fertilizers, leading to soil fertility decline and heightened chemical toxicity. This study investigates the isolation of phosphate-solubilizing bacteria (PSB) and nitrogen-fixing bacteria (NFB) from rhizosphere soil, with the aim of producing effective biofertilizers. Conducted at constructed wetland the research identified seven distinct bacterial isolates capable of enhancing soil NPK values. These isolates demonstrated resilience across varying environmental conditions, including temperature, salinity, and pH levels. Results from constructed wetland studies and in vitro assays indicated that these bacteria effectively improve soil health and nutrient availability, showcasing their potential as sustainable alternatives to chemical fertilizers. The implications of this research extend to restoring nutrient cycles and enhancing plant growth in diverse agricultural settings, particularly in regions facing soil degradation. Future applications of these biofertilizers may support sustainable farming practices while promoting entrepreneurial opportunities in agribusiness.

INTRODUCTION - To achieve optimal plant growth, it is essential that nutrients are present in sufficient and balanced quantities (Chen, 2006). Soil infertility is a major constraint on crop yields, particularly affecting resource-poor farmers in developing nations. Restoring soil fertility is crucial for these farmers to benefit from improved crop varieties and agricultural practices. This can be effectively accomplished through Integrated Soil Fertility Management (ISFM), which focuses on nutrient management, biological nitrogen fixation (BNF), and input efficiency (Vlek & Vielhauer, 1994). Biofertilizers are vital components of integrated nutrient management, offering eco-friendly and cost-effective solutions for enhancing soil productivity and sustainability. These products, which contain live microorganisms, improve nutrient availability by converting essential elements like nitrogen and phosphorus into forms that plants can absorb (Rokhzadi et al., 2008). They also promote plant growth and protect against pests and diseases (El-Yazeid et al., 2007). The history of using microbial inoculants dates back generations, beginning with small-scale compost production that demonstrated their effectiveness in improving soil health (NIIR Board, 2004; Abdul Halim, 2009). In Malaysia, the production of microbial inoculants began in the late 1940s, reaching a peak in the 1970s, largely influenced by research on Rhizobium for legumes (Abdul Halim, 2009). While biofertilizers are seen as potentially expensive and slower acting compared to chemical fertilizers, their renewable nature and lower production costs from abundant substrates present significant advantages. However, challenges such as short shelf life, environmental sensitivity, and transportation issues still need to be addressed for effective use (Rahim, 2002). The production of biofertilizers involves several critical factors, including the growth profile of microbes, their optimal conditions, and the formulation of the inoculum. The process typically consists of six key steps: selecting active organisms, isolating target microbes, choosing

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appropriate methods and carrier materials, determining propagation methods, and conducting prototype and large-scale testing. Active organisms, such as nitrogen fixers, potassium solubilizers, and phosphorus solubilizers, must be selected based on their roles in plant growth (Gupta, 2004). Carrier materials play a vital role; they should be non-toxic, moisture-retaining, and able to be sterilized, often using methods like autoclaving or gamma irradiation. Effective microorganisms include Rhizobium, which forms symbiotic relationships with legumes, and various bacteria and fungi that help solubilize phosphorus and potassium (Gupta, 2004). Biological nitrogen fixation (BNF) is crucial for converting atmospheric nitrogen into forms usable by plants. Free-living bacteria, such as Azotobacter and Azospirillum, can fix nitrogen without symbiosis, while others like Rhizobium rely on plant interactions to effectively enhance nitrogen availability (Gothwal et al., 2007; Kannaiyan, 2002). The application of biofertilizers, including combinations of bacterial species, has been shown to improve crop yields, nutrient uptake, and overall plant health, making them an essential tool for sustainable agriculture (Mohammadi et al., 2010). Bacteria are generally more effective than fungi in solubilizing phosphorus, with phosphate solubilizing bacteria (PSB) constituting 1-50% of the microbial population in soil, compared to only 0.1-0.5% for phosphorus solubilizing fungi (PSF) (Alam et al., 2002; Chen et al., 2006). In northern Iranian soils, PSB represented approximately 88% of the total phosphate solubilizing microorganisms (Fallah, 2006). Key PSB include genera such as *Pseudomonas*, *Bacillus*, and *Rhizobium*, while some fungi, like *Penicillium* and *Aspergillus*, also contribute to phosphate solubilization (Whitelaw, 2000). Microorganisms enhance phosphorus availability to plants through various mechanisms, including the secretion of organic acids that lower soil pH, facilitating the solubilization of bound phosphates (Sundara Rao & Sinha, 1963). The use of PSB can significantly reduce the need for chemical fertilizers; for instance, sugarcane yield can match that of full phosphorus applications when inoculated with phosphor-bacteria (Kathiresan et al., 1995). Furthermore, combined applications of biofertilizers and reduced chemical fertilizers have demonstrated improved crop yields and nutrient efficiency (Habibi et al., 2011).

Methodology

Sampling sites and Sample collection

In this study four sampling sites were constructed wetlands. The Samples were collected in a 5L pre-sterilized plastic container bottles. During sampling, sample bottles were rinsed three times with sampled water before filling the bottles to the brim from depths of 1 m below the surface of each designated sampling sites. The temperatures and pH of the samples were measured at the timeof sampling site using portable thermo meter and pH meter, respectively. The entire sample were to avoid any alteration in physico-chemical properties, all the samples were stored and carry in ice coldcondition (Osuolale and Okoh, 2015; Igbinosa and Okoh, 2009).

Collection of samples collected from constructed wetlands

A total of 20 samples collected for screening purpose. All the samples were collected in pre-sterilized plastic zip bags and B.O.D bottles and labeled properly. The temperature and pH were noted immediately at the site (pocket pH meter, Model No. 335) and an insulated ice-box was used to transfer the samples from collection point to workstation at Microbiology Laboratory.

Isolation of bacterial strain(s) from samples collected from constructed wetlands

Isolation of bacterial strain(s) was done by serial dilution method. The waste water was serially diluted in normal saline (0.85% NaCl) up to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} to 10^{-6} dilution. Diluted samples were spread on Nutrient Agar medium (NAM) plate and incubated at 37°C for 24 h. After the incubation, plates were observed for bacterial growth. Morphologically different bacterial colonies were isolated and NAM sub-cultured on plate. The Culture was maintain on NAM slant and stored at 4°C (Dhall *et al*., 2011; Krieg and Holt, 1984; APHA, 1998).

Determination of nitrate by phenoldisulphonic method: Nitrate reacts with phenoldisulphonic acid (PDA) to produce a nitro derivative, which in alkaline solution develops a yellow colour.

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Phosphate (reactive, ortho) - Stannous Chloride Method- In an acidic solution, ortho-phosphate reacts with ammonium molybedate to form molybedophosphoric acid, which is then reduced by stannous chloride to the intensely colored molybdenum blue. The resulting blue color is directly proportional to the phosphate concentration. Results are expressed in ppm (mg/L) phosphate as PO_4 or P. To convert results from ppm PO_4 to ppm P, divide by 3.06.

Analysis of Potassium Permanganate solution with Sodium Thiosulphate- Sodium thiosulphate solution is standardized against potassium dichromate in presence of hydrochloric acid and potassium iodide. Potassium dichromate oxidizes the iodide ion in acidic medium to equivalent amount of iodine. The iodine formed in the reaction oxidizes sodium thiosulphate giving sodium tetrathionate ion and the end point is detected by starch solution.

Inoculum preparation

To study the biodegradation efficiency, a loop full of 24 h old culture was transfer in 50 ml medium flask and incubated at 150 rpm for 24h at 37^0C. The growth of bacterial isolate was observed spectrophotometrically at 600 nm (OD_{600} ~ 2.0) and bacterial cell number was counted using McFarland 0.5standard. Activity growing cultures were harvested at 10,000 rpm for 5 min at 4^0C and cell pellet was washed with 50mM phosphate buffer, pH 6.8. After words, cell pellet was suspended in 50mom phosphate buffer (pH 6.8) till optical density were reached up 1ml, 2ml, 3ml, 4ml, 5ml (Wayne, 2009; Porwal *et al*., 2015).

Screening of bacterial isolates for NPK

Isolated bacterial strain was accessed to effect NPK value in broth and waste water. Bacteria increasing NPK value in constructed wetland was selected for further study. NPK analyses was done by aforementioned techniques

Compatibility analysis for the selected bacterial isolate(s)

The compatibility analysis of selected efficient bacterial isolates was investigated according to the method of Shinkafi *et al.* (2016) to this study. Mueller Hinton Agar (MHA) plates were swabbed with one of the selected microbe. Four to seven wells were cut and 10 µl of the culture supernatant (after 24-72 hrs of incubation) of the other organisms selected were added to the well. The test was repeated by changing the swabbed bacteria with the 10 selected cultures used in the study.

Identification The bacterial isolates exhibited having maximum waste water pollution reduction efficiency were identified on the basis of morphological, cultural and biochemical characteristics (Mac-Faddin, 1980) with the help of Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984) and Probabilistic Identification of Bacteria (PIB) computer kit (Bryant, 2003).

Result

Isolation and screening of bacterial isolates for enhancement of biofertilizers ability.- Total of 60 bacterial isolates were obtained from samples from constructed wetlands were collected during sampling procedure. All the isolates were enriched and incubated under similar growth conditions. Substantial N,P,K value of soil and water was observed .

Identification of bacterial isolates enhancing N, P, K value- On the basis of morphological studies different bacterial strains was selected for identification and isolation of rhizobium from root nodules showed similar morphological characteristics on nutrient agar media

Table 1: External morphological study of Rhizobia bacterial colony

plates	colony	colour	shape
10^{-2}	1	White,transculent	Circular
10^{-4}	2	White,transculent	Granular

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10^{-6}	3	White,transculent	Granular
10^{-8}	4	White,transculent,gummy	Granular

Table 2: Biochemical test of *Rhizobium* spp. strain

tests	*Rhizobium spp.*
Methyl red	+(strain 1,2,3,4)
Vp test	- (strain 1,2,3,4)
Catalase test	+ (strain 1,2,3,4)
Starch hydrolyses	- (strain 1,2,3,4)
Hydrogen sulphide	- (strain 1,2,3,4)
Triple sugar(glucose, sucrose, maltose)	+ (strain 1,2,3,4)

Isolation of bacterial strain for phosphate solubilization was done and after identification following strains showed the ability of phosphate solubilization among which *Pseudomonas* and *Bacillus* showed highest SI as 2.23 and 2.08 respectively followed by *Rhizobium* at 2.11.

Table 3: Phosphate solubilizing activites of six potent bacterial isolates

Sr.No.	Isolates	Colony measurement (cm)	Zone measurement(cm)	Solubilization index(SI)
1	*Pseudomonas sp.*	1.3	1.6	2.23
2	*Bacillus sp.*	2.0	2.3	2.15
3	*Aspergillus*	0.6	0.6	2.0
4	*Bacillus sp.*	1.2	1.3	2.08
5	*Azotobacter*	1.2	1.3	2.08
6	*Rhizobium*	1.9	2.1	2.11

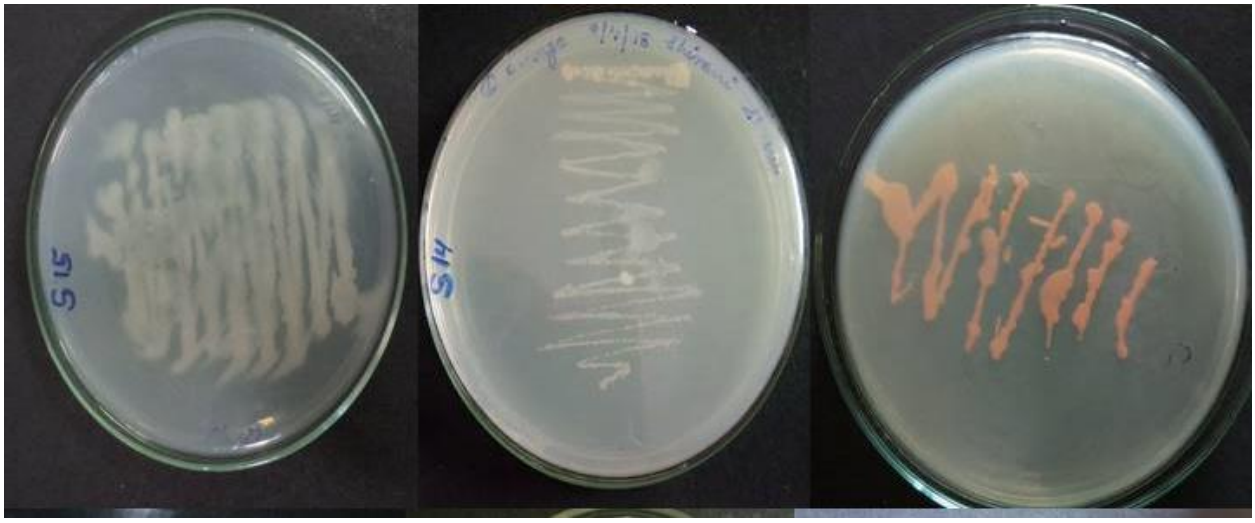

Figure 1 Isolation of Morphologically different potent bacterial strain

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Figure 2 Antagonistic activities of isolated bacteria

Table 4- **Morphological and Biochemical characterization of the isolates**

s.no.	Characteristics	A1	A2	A3	A4	A5
1	Gram reaction	-ve	+ve	-ve	+ve	-ve
2	Shape	Rods	Rods	Rods	Rods	Rods
3	Pigments	+	-	+/-	-	+/-
4	H_2S production	+	-	+	-	-
5	Indole	-	-	+	-	+
6	Methyl red	-	-	+	-	+
7	Vogues proskauer	-	+	+	+	+
8	Citrate utilization	+	+	-	+	+
9	Nitrate reduction	+	+	+	+	+
10	Starch hydrolysis	+	+	+	+	+
11	Gelatin hydrolysis	-	+	-	+	-
12	Lactose fermentation	-	+	+	+	+
13	Sucrose fermentation	+	+	+	+	+
14	Mannitol fermentation	-	+	+	+	+

Analysis of N,P,K value of various soil sample

1) Assessment of N, P, K value of various samples collected from constructed wetlands

Determining the nutrient concentration for nitrate, phosphate and potassium can revealed how a soil (constructed wetland) is functioning in regards to its intended use in crop production. Liquid sample both concentrated and diluted and solid samples processed (fine) and unprocessed was collected showing phosphate value from 0.1- 0.6 mg kg^{-1}, same samples

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showed nitrate value concentration from 0.2- 1.3 mg kg^{-1} and the same samples showed potassium value from 0.7-0.5 mg kg^{-1}.

Table-5 NPK analysis of samples collected from constructed wetlands

N mg kg^{-1}.	P mg kg^{-1}.	K mg kg^{-1}.
0.46	0.344	0.46
0.535	0.341	0.87
0.615	0.310	0.56
0.310	0.047	0.87
0.078	0.085	0.34
0.063	0.084	0.13
0.105	0.057	0.42
0.112	NA	0.43
NA	NA	0.34
NA	NA	0.23

Growth curve study of selected bacterial isolates

A2 Bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A2 strain sudden increase in growth was observed that 12hour showing log phase (0.167).Another increase at 22hr (0.262) showed continuity of log phase revealing 12 hr. to 22hr of active bacterial metabolism phase.

A4 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A4 strain sudden increase in growth was observed that 6hour showing log phase (0.305).Another increase at 12hr (0.521) showed continuity of log phase revealing 6^{th} hr. to 12^{th} hr of active bacterial metabolism phase.

A5 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A5 strain sudden increase in growth was observed that 6^{th} hour showing log phase(0.235).Another increase at 16^{th} hr (0.588) showed continuity of log phase revealing 6^{th} hr. to 16^{th} hr of active bacterial metabolism phase.

A11 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A11 strain sudden increase in growth was observed that 12hour showing log phase(0.220).Another increase at 22hr (0.360) showed continuity of log phase revealing 12 hr. to 22hr of active bacterial metabolism phase.

A6 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A6 strain sudden increase in growth was observed that 10^{th} hour showing log phase(0.146).Another increase at 16^{th} hr (0.221) showed continuity of log phase revealing 10 hr. to 16hr of active bacterial metabolism phase.

A7 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A7 strain sudden increase in growth was observed that 10^{th} showing log phase(0.155).Another increase at 22hr (0.339) showed continuity of log phase revealing 10th hr. to 22th hr of active bacterial metabolism phase.

A3 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A3 strain sudden increase in growth was observed that 10hour showing log phase (0.147).Another increase at 16hr (0.210) showed continuity of log phase revealing 10 hr. to 16hr. of active bacterial metabolism phase.

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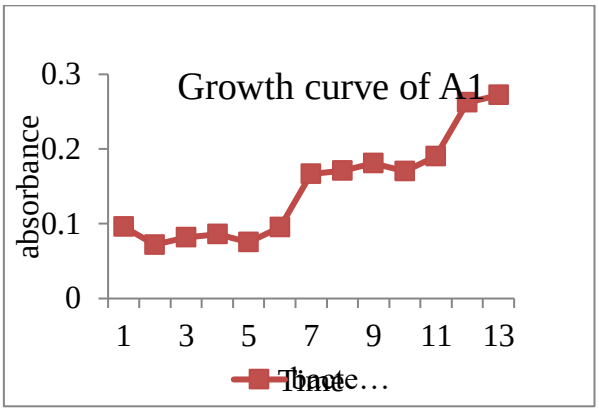
A12 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A12 strain sudden increase in growth was observed that 6 hour showing log phase (0.194).Another increase at 12hr (0.388) showed continuity of log phase revealing 6hr. to 12hr of active bacterial metabolism phase.

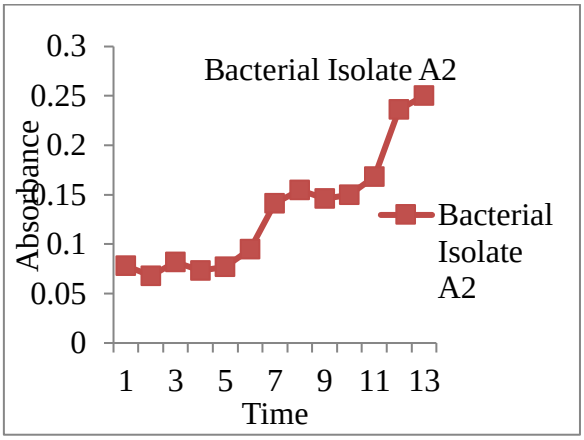


A8 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A8 strain sudden increase in growth was observed that 8hour showing log phase(0.174).Another increase at 14hr (0.281) showed continuity of log phase revealing 8hr. to 14 hr of active bacterial metabolism phase.

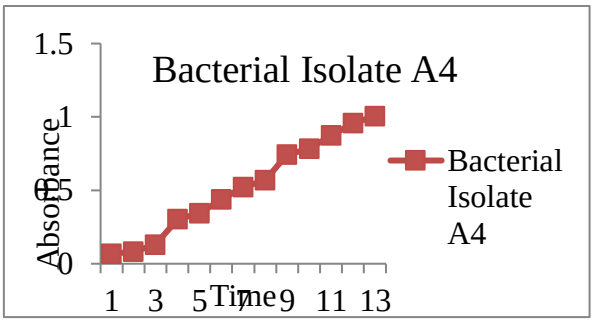
A13 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A13 strain sudden increase in growth was observed that 4th hour showing log phase(0.446)Another increase at 8thr (0.986))) showed continuity of log phase revealing 4th hr. to 8th hr. of active bacterial metabolism phase.



A14 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A14 strain sudden increase in growth was observed that 8th hr. showing log phase(0.780)).Another increase at 12th hr. (1.074) showed continuity of log phase revealing 8th hr. to 12th hr. of active bacterial metabolism phase.

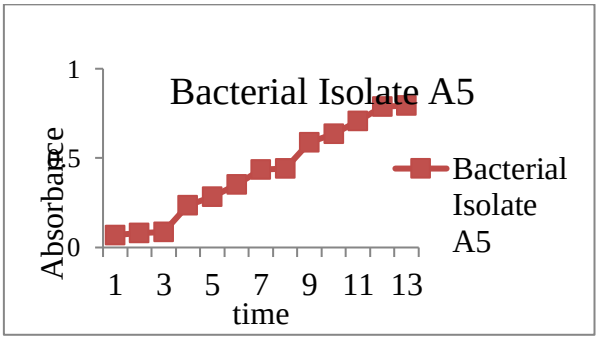


A15 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A15 strain sudden increase in growth was observed that 12th hr. showing log phase(0.441)).Another increase at 22th hr. (0.636) showed continuity of log phase revealing 12th hr. to 22th hr. of active bacterial metabolism phase.

A16 bacteria

Growth curve study of morphologically different bacterial strain was assessed for 24hour for A16 strain sudden increase in growth was observed that 4th hr. showing log phase(0.940)).Another increase at 14th hr. (2.680) showed continuity of log phase revealing 4th hr. to 14th hr. of active bacterial metabolism phase.



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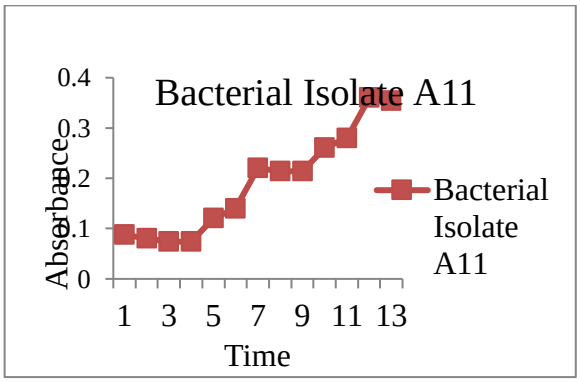

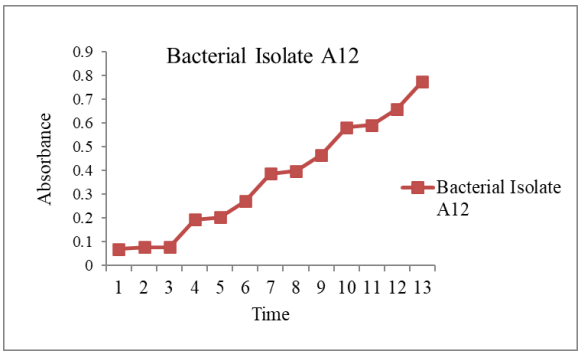

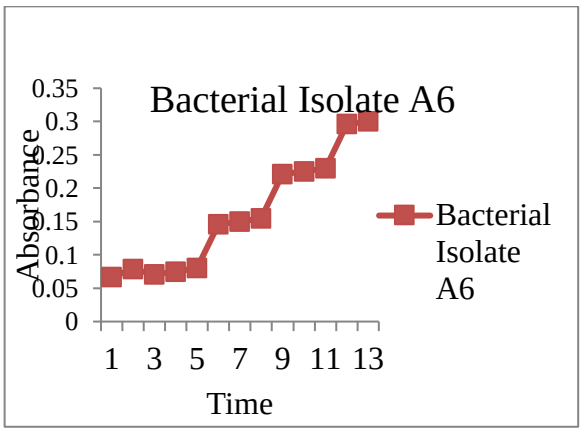

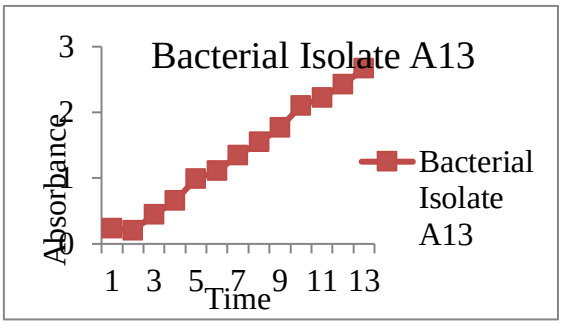

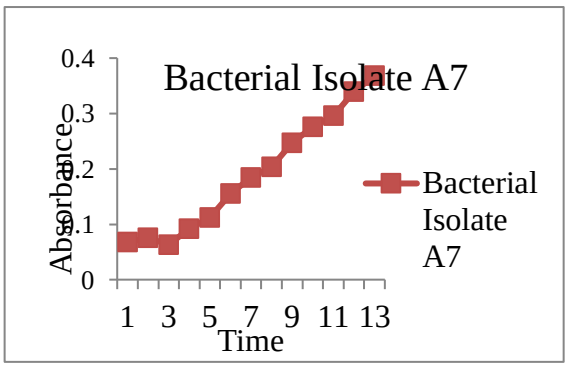

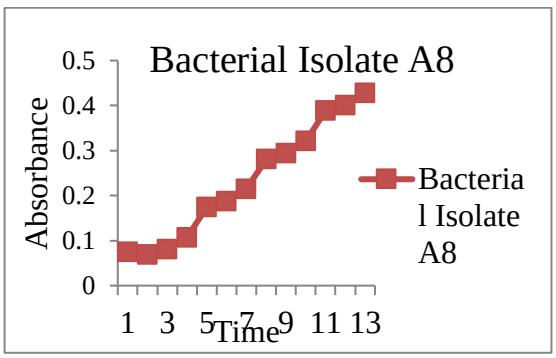

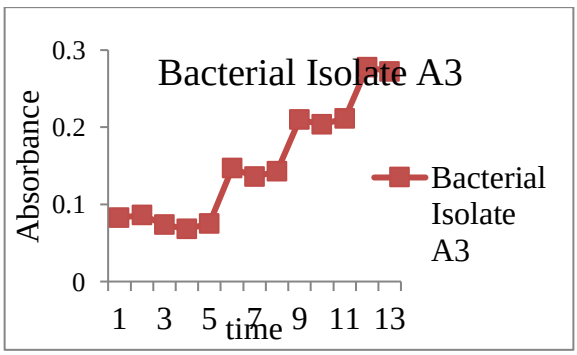

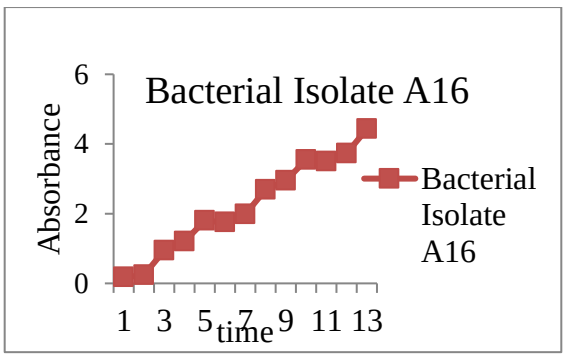

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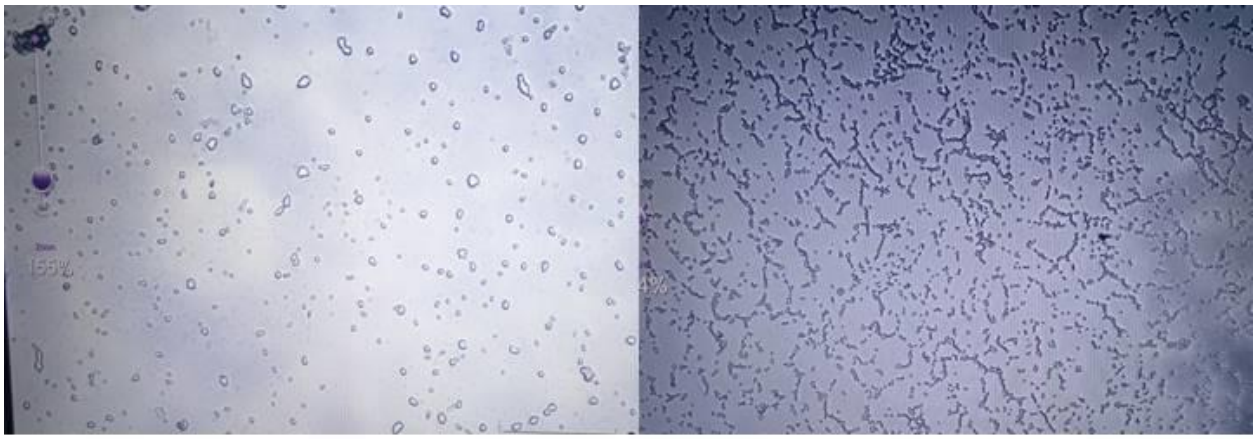
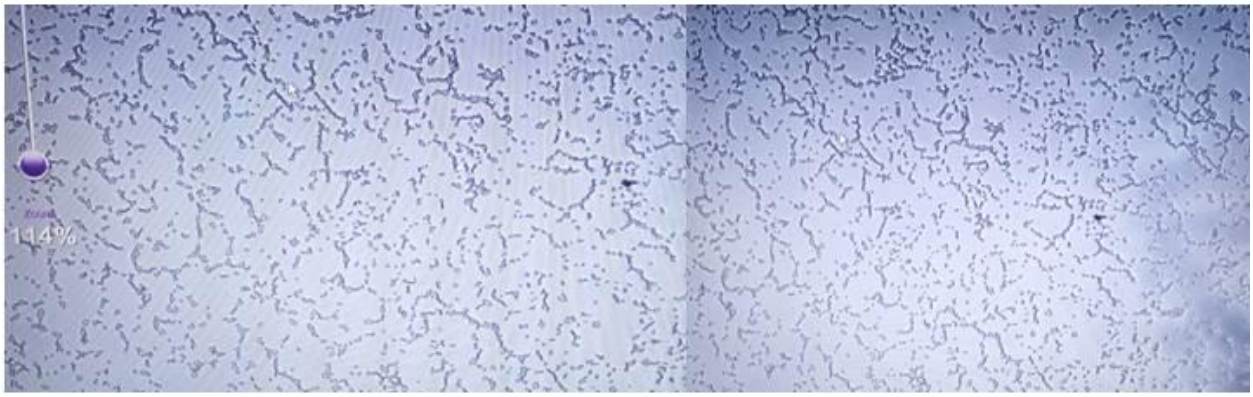


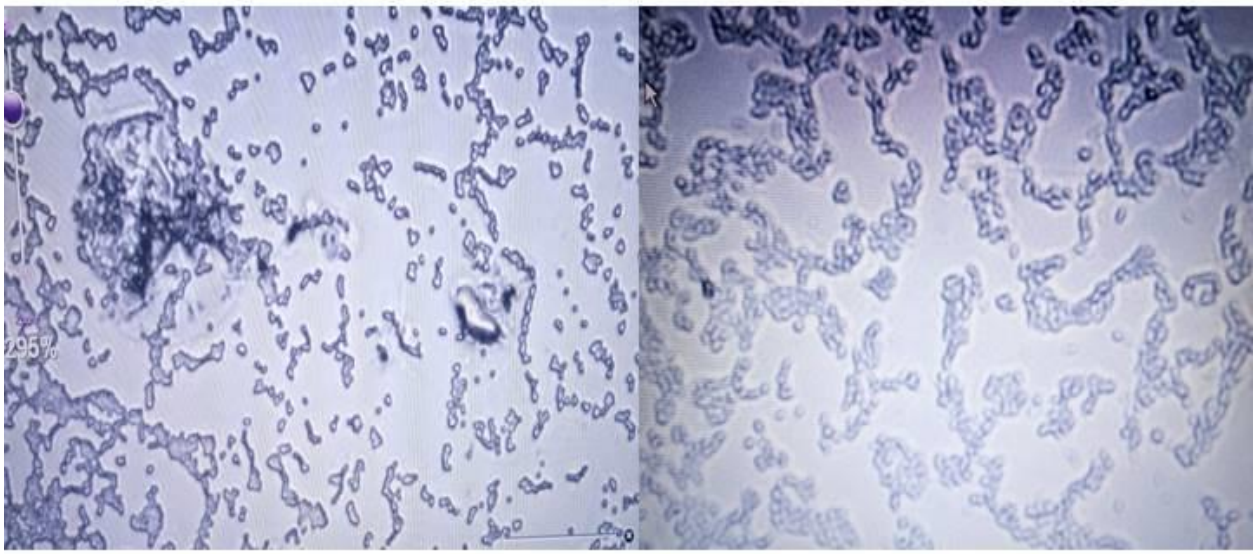
A1 – after two hour of inoculation

A2– after two hour of inoculation



A1 – after eight hour of inoculation

A2– after eight hour of inoculation



A1 – after twelve hour of inoculation

A2– after twelve hour of inoculation

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Evaluation of N,P,K value after enrichment by isolated bacterial strain

Biofertilizers potential of constructed wetland water sample used for plant growth was assessed with and without bacterial strain and was found that nitrogen content was raised from 0.3-0.6 ,phosphate content -0.1-0.5 and potassium content from 0.07 -0.3

Table: Solubilization of NPK by selected bacterial strain

Untreated sample			
Sample	N	P	K
Soil	0.650	0.540	0.348
Water	0.126	0.187	0.070

Treated with bacteria			
sample	N	P	K
A16	0.306	0.528	0.227
A9	0.221	0.462	0.233
A7	0.280	0.423	0.198
A10	0.510	0.402	0.216
A8	0.374	0.414	0.173

Untreated sample			
Sample	N	P	K
Soil	1.002	2.422	0.605
Water	0.314	0,260	0.210

Treated with bacteria			
Sample	N	P	K
A5	0.560	0.560	0.296
A11	0.530	0.720	0.496
A4	0.410	0.412	0.295
A3	0.705	0.660	0.400
A1	0.580	0.570	0.375

Discussion-

Recent advancements in agricultural practices have led to increased reliance on chemical fertilizers, resulting in declining soil fertility and rising chemical toxicity (Chen, 2000). Soil infertility is a significant constraint on global crop yields, and restoring soil fertility is crucial for optimizing the benefits of improved crop varieties and cultural practices (Mohammad & Sohrabi, 2012). Biofertilizers, which consist of living microorganisms, are essential components of integrated nutrient management. They offer eco-friendly and cost-effective alternatives to chemical fertilizers by enhancing nutrient availability through processes like nitrogen fixation and phosphorus solubilization (Rokhzadi et al., 2008). Microbial biofertilizers have gained recognition as effective alternatives to chemical fertilizers, promoting plant growth through mechanisms

such as biological nitrogen fixation, hormone synthesis, and increased nutrient availability (Fuentes-Ramirez & Caballero-Mellado, 2005). The application of these biofertilizers can improve crop yields by 20-30% and reduce reliance on chemical inputs, thus contributing to sustainable agricultural practices (Ghosh, 2007). The isolation and application of beneficial constructed wetland soil bacteria for biofertilizer production can enhance soil fertility and plant health, making them vital for achieving sustainability in agriculture. The study investigates the synergistic effects of rock-derived phosphorus (P) and potassium (K) fertilizers, along with co-inoculation of phosphorus-solubilizing bacteria (PSB) and potassium-solubilizing bacteria (KSB) (Hans et al., 2005). Biofertilizers, which include beneficial microorganisms, enhance soil fertility by fixing atmospheric nitrogen and solubilizing nutrients like phosphorus and potassium, ultimately improving crop yields. These microbial agents play essential roles in nutrient cycles and contribute to sustainable agriculture, particularly in regions where chemical fertilizer use leads to soil degradation and pollution (Ismail & Hasabo, 2000). Future research should focus on developing efficient biofertilizers that minimize chemical inputs while maximizing crop productivity and environmental sustainability. This includes selecting multifunctional biofertilizers, ensuring quality control, and studying their persistence in diverse soil environments (Safir, 1994).

Chemical used as biofertilizers has serious side effects on soil and plant. In this study we have isolated bacterial species and used them to enhance soil health. The main aim of this research paper is the isolation of Phosphate solubilizing bacteria and nitrogen fixing from constructed wetland water and soil and hence the production of bio fertilizer from it. Most soils are deficient in soluble forms of phosphorous and it is also a fact that the NPK is a very important macro nutrient for the growth of plants. Apart from nitrogen phosphorous and potassium plays a very important role in the plant metabolism such as cell division, growth and development, breakdown of sugar, and nuclear transport within the plants. NPK solubilizing bacteria can hence be utilized for the production of bio fertilizers which actually enhances the nutrient quality of soil. Seven morphologically distinguished bacterial isolates were isolated. These isolates were studied for their colony characteristics and were found to be Gram negative and positive in nature. These isolates could grow at a broad temperature range, tolerate high concentrations of salt and survive in varying pH concentrations. The isolates were checked for their ability to enhance the NPK values of soil these isolates if used as bio fertilizers can restore the soil's natural nutrient cycle and build soil organic matter, thus providing nutrients to the growing plants. These Nitrogen fixing bio fertilizers are very important for maintaining the soil fertility when applied to different soils. They are finally packeted to different hilly regions for application in soils. Finally these bio fertilizers with huge CFU numbers when mixed in soil increases the nutrient availability to the plants. Moreover these bio fertilizers can be used in a vast area of agribusiness which finally has a growth potential in an entrepreneur model.

Conflict of Interest – The authors have no conflicts of interest to declare

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