

## Enterobacter cloacae PNE2 As Promising Plant Growth Promoting Bacterium, Isolated From The Kadi Vegetable Market Waste, Gujarat

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Chemical fertilizer use in agricultural areas causes a variety of issues, including pollution, health risks, disruption of natural ecological nutrient cycles, and the loss of biological communities. In this case, chemical fertilizers, herbicides, and other supplements are replaced by plant growth promoting bacteria for sustainable agriculture. The present research work focus on the isolation of the plant growth promoting bacteria from the Kadi vegetable market waste. Derived from morphological, biochemical, and 16S rRNA gene sequence analysis the strain was identified as *Enterobacter cloacae* PNE2. The antibiotic susceptibility test indicated that the isolate was sensitive to all 22 antibiotics tested. The isolate *Enterobacter cloacae* PNE2 has multiple growth-promoting activities like N<sub>2</sub> fixation, phosphate, solubilization, potassium solubilization, phytohormone (Indole-3-acetic acid) production, EPS production, biopolymer degradation, and also possesses good seed germination ability. Quantitative analysis of nitrite production revealed the isolate *Enterobacter cloacae* PNE2 produced  $0.15 \pm 0.01 \mu\text{g/ml}$  nitrite. The Phosphate Solubilization Index (PSI) of the isolate was recorded  $3.58 \pm 0.08$  and the isolate released  $278.34 \pm 0.56 \mu\text{g/ml}$  phosphate in Pikovskaya's broth. The isolate *Enterobacter cloacae* PNE2 solubilized  $32.66 \text{ mg/l}$  potassium. The isolate *Enterobacter cloacae* PNE2 possesses IAA ( $48.49 \pm 0.05 \mu\text{g/ml}$ ) in presence of tryptophan and EPS ( $19.1 \pm 0.2 \text{ g/l}$ ) production ability. The isolate *Enterobacter cloacae* PNE2 was also found to degrade Cellulose, Pectin, and Xylan. Furthermore, the isolate *Enterobacter cloacae* PNE2 enhances seedling growth of *Trigonella foenum graecum* (fenugreek). Thus, the isolate *Enterobacter cloacae* PNE2 has significant plant growth promoting characteristics and can be applied in a bio-fertilizer formulation for sustainable agriculture.

**Keywords:** *Enterobacter cloacae* PNE2; Plant growth promoting bacteria; seed germination; *Trigonella foenum graecum*.

Agriculture plays a vital role in the economy in addition it is considered to be the backbone of the economic system for developing countries, but in the twenty-first century, the world's agricultural system is facing new challenges, such

as failing productivity and deterioration in the agroecosystem sustainability<sup>1,39</sup>. The world's population is currently around 7.9 billion, and it is expected to reach 8.5 billion by 2030<sup>40</sup>. India's population will also increase to 1403 million by

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2025, as compared to the present 1391 million (2020-2021). Agriculture will play an essential role in supplying increased food demands for the rising human population as result in greater demand for to use of chemical fertilizers and pesticides. Moreover, the frequent use of these chemical fertilizers will cause air and groundwater pollution through the eutrophication of water bodies, will also negatively affect soil fertility, lower agricultural productivity, damage soil, and cause biodiversity loss. Therefore, using chemical fertilizer and pesticides poses a major risk to the ecosystem as well as health issues for people <sup>2</sup>.

Plant growth promoting bacteria are used to replace these chemical fertilizers through a variety of mechanisms, including soil structure formation, organic matter decomposition, element recycling, mineral solubilization, plant growth regulator production, organic pollutant degradation, root growth stimulation, and soil fertility enhancement <sup>3</sup>.

There are numerous genera in the *Enterobacteriaceae* family that have plant growth promoting abilities for e.g. *Enterobacter*, *Erwinia*, *Klebsiella*, *Kluyvera*, *Pantoea*, and *Serratia* <sup>4</sup>.

*Enterobacter cloacae* is a gram-negative, short rod of the *Enterobacteriaceae* family <sup>5</sup>. Several strains of *Enterobacter cloacae* have been reported as plant growth promoters due to their multiple growth promoting activities like phosphate solubilization, nitrogen fixation, phytohormone production, exopolysaccharides production, 1-Aminocyclopropane-1- carboxylate deaminase (ACC) production, etc <sup>6</sup>. Thus, *Enterobacter cloacae* have the potential to contribute to the development of sustainable agricultural systems.

Nitrogen is the most vital element for plant growth and development. Approximately 78–79% of the available nitrogen (N) in the atmosphere do not directly absorb by the plants. Several plant growth promoting bacteria can able to fix atmospheric nitrogen dioxide into ammonia, which the plant may easily absorb<sup>7</sup>. *Enterobacter cloacae* HG-1 has nitrogen fixation ability<sup>7</sup>.

Phosphorus (P) is the second most important element for plant growth <sup>8</sup>. It has a key role in several physiological processes in plants, particularly photosynthesis, carbon metabolism, and membrane production. Additionally, it is essential for root elongation, proliferation and its

deficiency affects root structure <sup>9</sup>. Approximately 95 to 99 percent of phosphorus is found in soil in an insoluble form along with Fe, Zn, Al, and Ca. Only 1% to 2% of the phosphorus is taken by plants. Phosphate solubilizing microorganisms play a key role in phosphorus nutrition by transferring its availability to plants through solubilization and mineralization from inorganic and organic soil phosphorus. The majority of powerful phosphate solubilizing bacteria belong to bacteria such as *Pseudomonas*, *Enterobacter*, *Enterobacter cloacae* BAU3, and *Bacillus* <sup>10</sup>.

Potassium (K) is the third most important element for a plant. It is important for photosynthesis, activation of enzymes, and synthesis of protein. As more than 90% of potassium exists in the form of insoluble rock and silicate minerals, the concentration of soluble potassium is usually very low in soil <sup>1</sup>. Certain microorganisms use several biological processes to make potassium available from unavailable forms i.e. *Klebsiella variicola*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter aerogenes*, *Burkholderia cepacia*, *Microbacterium foliorum*, and *Pantoea agglomerans* <sup>11,2</sup>.

Furthermore, plant growth promoting bacteria are capable of synthesizing phytohormones (Indole-3 acetic acid). Indole acetic acid has various functions like cell division, stimulation of seed germination, pigment production, and synthesis of metabolites. Numerous *Enterobacter* spp. having the potential to produce indole acetic acid (IAA) such as *E. cloacae* H3, *E. cloacae* NII-0931, *E. cloacae* MSR1, *E. cloacae* UW 5, *E. asburiae*, *E. cancerogenus* <sup>13</sup>.

Several strains of plant growth promoting bacteria can able to produce exopolysaccharides (EPS). The EPS-producing plant growth promoting bacteria significantly enhance the volume of soil macropores and the rhizosphere soil aggregation, resulting in increased water and fertilizer availability to inoculated plants <sup>14</sup>. Exopolysaccharides can be produced in significant quantities by the bacterial strain *Enterobacter cloacae* <sup>15</sup>.

In the present research work, we have isolated the *Enterobacter cloacae* PNE2 from the vegetable market waste and characterized its plant growth promoting capability based on N<sub>2</sub> fixation, phosphate solubilization, potassium solubilization, IAA production, EPS production,

biopolymer degradation, and seedling growth of *Trigonella foenum graecum* (fenugreek) plant. Based on obtained results, we propose to use the indigenous isolate *Enterobacter cloacae* PNE2, as a biofertilizer.

## MATERIALS AND METHODS

### Sample collection and isolation of the *Enterobacter cloacae* PNE2 from the vegetable market waste

For the bacterial isolation mixed consolidated vegetable waste was collected from the Kadi vegetable market in Mehsana distinct from Gujarat. The sample was collected on a random basis and transferred into the laboratory. A serial dilution method was carried out to isolate bacteria from vegetable market waste. 1 ml of a separated liquid component collected from the vegetable market waste was added to 9 ml sterilized water and then serially diluted up to 10<sup>6</sup>. Initially, the isolation was done using Ashby's Mannitol agar. Spread 0.1 ml of each diluted sample onto Ashby's Mannitol agar and the plate was incubated at 37±2 °C for 48 hours. The colonies that grow on Ashby's Mannitol agar are thought to have the potential for nitrogen fixation. The colony was then isolated and purified depending on the distinctive colony morphology for further study<sup>14</sup>.

### Identification of the isolate

#### Morphological and biochemical identification

The Isolate was identified based on Gram-staining, morphological characteristics, and biochemical test using the HI-Assorted biochemical test kit (KB003) of HI-media Laboratories, Mumbai, India<sup>14</sup>.

#### Molecular identification

Molecular identification of the isolate *Enterobacter cloacae* PNE2 was done by 16S rRNA partial gene sequencing analysis respectively. Fragment of 16S rRNA gene was amplified by PCR. Takara (EmeraldAmp® GT PCR Master Mix) was used for the amplification of DNA templates. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 27 F & 1492 R universal primers using BigDye™ terminator v3.1 cycle sequencing kit<sup>16</sup>. v 6.0 was used for sequence analysis from the electropherogram

generated by the 3500XL Genetic Analyzer. The 16S rRNA sequence was used to carry out BLAST with the database of NCBI GenBank.

### Phylogenetic Analysis

The Neighbor-Joining method was used to deduce the evolutionary history<sup>17</sup>. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches<sup>18</sup>. The evolutionary distances were computed using the greatest composite likelihood method, which is indicated the units of several base substitutions per site<sup>19</sup>. This analysis involved 8 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 842 positions in the final dataset. Evolutionary analyses were conducted in MEGA11<sup>20</sup>.

### Antibiotic Susceptibility test

The disc diffusion method was used to check the antibiotic susceptibility of the strain *Enterobacter cloacae* PNE2. The method involved spreading the 0.1ml of overnight grown culture (set the O.D 0.5 at 540 nm)<sup>21</sup> on a nutrient agar plate and placing antibiotic multidisc on the surface of the plate, incubating the plate at 37±2 °C for 24 hrs. Antibiotic susceptibility test was conducted using combi disk for Gram Negative bacteria from Hi Media Laboratories<sup>14</sup>. The zone of inhibition was measured<sup>22</sup>.

### Plant growth-promoting characterization of the isolate *Enterobacter cloacae* PNE2

The *Enterobacter cloacae* PNE2 was further screened for plant growth promoting traits, like nitrogen fixation, phosphate solubilization, potassium solubilization, IAA production, EPS production, biopolymer degradation, and seed germination ability.

### Determination of nitrite nitrogen

Quantitative determination of nitrite nitrogen was carried out by estimating nitrite nitrogen. 3 ml of 24 hrs old culture (O.D adjusted to 0.5 at 540 nm) was inoculated in 100 ml of Ashby's N-free liquid medium. The inoculated flasks were incubated at room temperature in static conditions. Approximately 2ml broth was harvested at the interval of 24 hrs. Following the appropriate incubation period, the culture broth was centrifuged for 10 minutes at 10,000 rpm<sup>14</sup>. Nitrite

nitrogen production in the broth was examined at an interval of 24 hrs of incubation by standard method<sup>23</sup>.

#### **Determination of phosphate solubilization activity**

The isolate *Enterobacter cloacae* PNE2 was spot inoculated on Pikovskaya's medium and the plate was incubated at  $37\pm 2^\circ\text{C}$  for 48 hrs. After the incubation periods measured the Phosphate Solubilisation Index (PSI) by the following equation<sup>22</sup>.

$$\text{PSI} = (\text{colony diameter} + \text{halo zone}) / \text{colony diameter}$$

Quantitative estimation of phosphate solubilization was carried out by using Pikovskaya's liquid medium abundant with 0.5% tricalcium phosphate. Selected isolate *Enterobacter cloacae* PNE2 was inoculated in Pikovskaya's broth and incubated for 7 to 9 days at  $37\pm 2^\circ\text{C}$  under shaking conditions at 120 rpm. At an interval of 24 hrs, approximately 2ml of broth was harvested and centrifuged for 10 min at 10000 rpm. The vanadomolybdo phosphoric acid method was used to estimate the amount of phosphorous in the broth was estimated from the supernatant<sup>24</sup>. A simultaneous change in the pH was also recorded from the supernatant by using eqiptronic digital pH meter.

#### **Determination of Potassium Solubilization activity**

Qualitative determination of Potassium Solubilization was carried out by using modified Aleksandrow agar medium + bromothymol blue. The isolate *Enterobacter cloacae* PNE2 was spot-inoculated onto the modified Aleksandrow agar medium, and incubate on the plate for 24 hrs at  $37\pm^\circ\text{C}$ <sup>14</sup>. Khandeparkar's selection ratio method was used to measure the formation of clear halos surrounding the colony<sup>25</sup>.

$$D/d = \text{Diameter of zone hydrolysis} / \text{Diameter of growth}$$

#### **Quantitative estimation of Potassium Solubilization activity**

Quantitative determination of potassium solubilization was carried out by estimation of K released from broth supplemented with 0.5 % Feldspar. Three ml of 24 hrs old culture (O.D adjusted to 0.5 at 540 nm) was inoculated in 100 ml of GYF (Glucose Yeast extract feldspar) broth<sup>26</sup>. The inoculated flasks were incubated in an

environmental shaker at 120 rpm. The amount of K released in the broth was examined at intervals of 7, 15, and 21 days of incubation. At the interval of the 7 days, approximately 5ml broth was harvested and centrifuged for 10 min at 10,000 rpm. Take supernatant from the centrifuge tube and estimate the potassium released from the broth by the flame photometric method.<sup>14</sup>

#### **Determination of Indole acetic acid (IAA) production**

The IAA production was determined qualitatively using Luria agar mixed with 0.06% sodium dodecyl sulphate and 1% glycerol.

Qualitative determination for the IAA production was detected using Luria agar supplemented with 0.06% sodium dodecyl sulphate and 1% glycerol. The overnight grown culture of the isolate was spot inoculated in each plate. The plates were layered immediately with a sterile disc of Whatman No.1 filter paper. After 48hrs, the filter paper disc was removed from the plates and treated with Salkowaski's reagent (2% of 0.5M  $\text{FeCl}_3$  in 35% perchloric acid) by soaking in a petridish containing the reagent. The reaction was allowed to proceed until the adequate color was developed<sup>27</sup>.

#### **Quantitative estimation of IAA production**

For the quantitative estimation of IAA production, three ml of 24 hrs old culture of the isolate *Enterobacter cloacae* PNE2 (O.D adjusted to 0.5 nm) was inoculated in 100 ml Luria broth supplement With 0.1% tryptophan or without tryptophan and the flask was incubated at  $37\pm 2^\circ\text{C}$  for 7 days at under shaking condition at 120 rpm. Salkowski's reagent was used to estimate the amount of IAA production in 1 ml of the supernatant after centrifuging 2 ml of broth for 10 min at 10,000 rpm<sup>28</sup>.

#### **Quantitative estimation of EPS production**

The isolate *Enterobacter cloacae* PNE2 for the production of EPS was measured using the standard method. For quantitative estimation, the overnight grown culture of the isolate was inoculated in yeast extract medium supplemented with 5% sucrose and incubated at  $37\pm 2^\circ\text{C}$  for 5 days on a shaker at 120 rpm. At the interval of 24 hours, 10 ml of broth was taken in a centrifuge tube and centrifuged at 10,000 rpm for 10 min. The supernet was collected from each centrifuge tube and Three-time increased cooled acetone was

added. A slimy precipitate that contained EPS was removed from the mixture and gathered on a pre-dried filter paper. At a temperature of 50 °C, the precipitates were left to dry overnight. After drying for the entire night, the filter paper was reweighed. The amount of EPS produced was determined by increasing the filter paper weight.<sup>24,14</sup>

#### Determination of potential for biopolymer degradation

The potential of the isolate *Enterobacter cloacae* PNE2 for degradation of cellulose, pectin, and xylan was carried out on a solid medium supplemented with 1% CMC, 1% Pectin, and 1% beech wood Xylan respectively as substrate with pH 6.8. The isolate was spot inoculated on basal mineral salt medium with CMC/Pectin/Xylan and plates were incubated at 37±2°C till the development of colonies on the medium<sup>29</sup>. The zone of solubilization was measured after flooding with 1% iodine solution on the medium. The Khandeparkar method was used to calculate the zone ratio. The Khandeparkar method was used to measure the zone ratio<sup>25</sup>.

$D/d = \text{Diameter of zone of hydrolysis} / \text{Diameter of growth}$

#### Seed germination assay

Fenugreek seed was used for seed germination assay. Seed germination assay was performed by soil method<sup>24</sup>. All of the chosen seeds were surface sterilized for 90 seconds with 1% NaOCl then immersed for 30 seconds in 70%

ethanol, rinsed twice with sterile distilled water, and air drying under laminar airflow<sup>16</sup>. A total of 50 Fenugreek seed was soaked for 15 minutes in 10 ml overnight grown bacterial culture *Enterobacter cloacae* PNE2 (adjust optical density 0.5 at measured at 540 nm which contain 10<sup>8</sup> cells /ml). Two controls were used for this study. i) Control 1<sup>st</sup> (primed control): 50 seeds were soaked in 10ml sterile distilled water for 15 minutes. ii) Control 2<sup>nd</sup> (unprimed control): 50 dry seeds were directly used in sowing without any treatment. The experiment was set up in black plastic bags which contain 300gm soil. Ten seeds were sown in each bag. The experiment was monitored for up to 7 days and the number of seeds germinated, germination index, seed vigour index, and plumule length were recorded. After recording the results for 7 days, the plants were uprooted, and measured the shoot lengths, root lengths, wet weight, and dry weight of the *Trigonella foenum graecum* (fenugreek) plant<sup>24</sup>.

#### Statistical analyses

The result was shown as the mean± standard deviation (SD) for all experiments, which were carried out in triplicate<sup>40</sup>. T-test two-sample with unequal variances was used to compare mean values, and significance was accepted at Pd<sup>77</sup> 0.05 level<sup>42</sup>. Mean values were compared using a two-sample T-test with unequal variance, and significance was accepted at Pd<sup>77</sup> 0.05 level<sup>43</sup>.

**Table 1.** Biochemical characterization of the isolate *Enterobacter cloacae* PNE2

Biochemical Test	Results	Biochemical Test	Results
ONPG (O-Nitrophenyl-β-D-Galactopyranoside)	+	Pectinase	+
Lysine	-	Xylanase	
Ornithine utilization	-	Arabinose	+
Phenylalanine	-	Xylose	
Nitrate reduction	+	Adonitol	-
H <sub>2</sub> S production	-	Rhamnose	+
Citrate utilization	+	Cellobiose	+
VP (Voges Proskauer)	+	Melibiose	-
MR (Methyl Red)	-	Saccharose	+
Indole	-	Raffinose	+
Esculin hydrolysis	+	Trehalose	+
Urease	+	Glucose	+
Lipase	+	Lactose	+
Amylase	+	Sorbitol	-
Cellulase	+	Malonate	+

## RESULTS AND DISCUSSION

### Isolation and identification of strain PNE2

The strain was isolated from the liquid content of vegetable market waste. The ability to fix atmospheric nitrogen was tested by inoculating the isolate on Ashby's Mannitol agar. The isolates grow on nitrogen-deficient media, indicating that

the isolate *Enterobacter cloacae* PNE2 was able to fix atmospheric nitrogen from the environment. The isolate was purified and preserved in the same medium for further study. Figure 1 depicts the data of cultural characteristics on Ashby's Mannitol agar medium and Morphological characteristics of the selected isolate. On Ashby's Mannitol agar, isolate formed non pigmented, medium, round, entire

**Table 2.** Antibiotic susceptibility test of *Enterobacter cloacae* PNE2

Antibiotic Disk	µg/ml	Zone of inhibition (mm)	Antibiotic Disk	µg/ml	Zone of inhibition (mm)
Tetracycline	30	5	Ceftazidime	30	20
Streptomycin	10	15	Cefepime	30	12
Nitrofurantoin	300	10	Cephalothin	30	15
Kanamycin 30	30	9	Cefotaxime	30	20
Co-Trimazine	25	5	Imipenem	10	15
Carbenicillin	100	22	Meropenem	10	18
Amikacin	30	10	Ciprofloxacin	5	20
Ofloxacin	25	21	Tobramycin	10	22
Co-Trimoxazole	25	18	Moxifloxacin	5	20
Gentamicin	10	10	Sparfloxacin	5	15
Ceftriaxone	30	20	Netllin	30	20

**Table 3.** Characterization of nitrite nitrogen by *Enterobacter cloacae* PNE2:

	8 <sup>th</sup> day	9 <sup>th</sup> day
Nitrite	0.15±0.01 µg/ml	0.08 ±0.02 µg/ml

glossy, mucoid, opaque, convex colony. According to its morphology, the isolate was a Gram-negative short rod.

### Biochemical characterization

Results depicted in Table 1 show the data of biochemical characterization of the

**Table 4.** Qualitative determination of soluble Potassium by *Enterobacter cloacae* PNE2.

Days	Colony size (mm)	Zone size (mm)	Zone ratio (mm)
1	6	10	1.6
2	8	15	1.8
4	10	21	2.1
7	12	23	1.76

**Table 5.** Potential for biopolymer degradation

Colony size	Zone of hydrolysis (mm)							
	Cellulose		Pectin			Xylan		
Colony size	Zone size	Zone ratio	Colony size	Zone size	Zone ratio	Colony size	Zone size	Zone ratio
10	55	5.5	7	65	9.2	12	20	1.6

isolate *Enterobacter cloacae* PNE2. Out of thirty biochemical tests performed, the isolate shows a positive result for 16 tests and a negative result for 9 tests. Out of 13 sugars the isolate fermented 10 sugars. Only 3 sugars melibiose, adonitol, and sorbitol are not utilized by the isolate. The isolate tested positive for ONPG, Urease production, Nitrate reduction, Citrate utilization, the Voges Proskauer test, and Esculin hydrolysis, showing that it has a wide metabolic range. In addition, lysine, ornithine utilization, phenylalanine,

H<sub>2</sub>S generation, Methyl red, and Indole are all negative. The isolate was able to produce the enzymes like urease, lipase, protease, cellulase, pectinase, and xylanase indicating their biopolymer degradation ability. Out of 24 biochemical tests performed the isolate *Enterobacter cloacae* PGLO9 was shown to be 21 positive results i.e xylose, maltose, galactose, raffinose, trehalose, melibiose, L- arabinose, mannose, insulin, glycerol, inositol, sorbitol, mannitol, α-methyl-D-glucoside, glucoside, cellobiose, ONPG, esculin hydrolysis,

**Table 6.** Effect of *Enterobacter cloacae* PNE2 on seedling growth of fenugreek plant up to 7<sup>th</sup> days

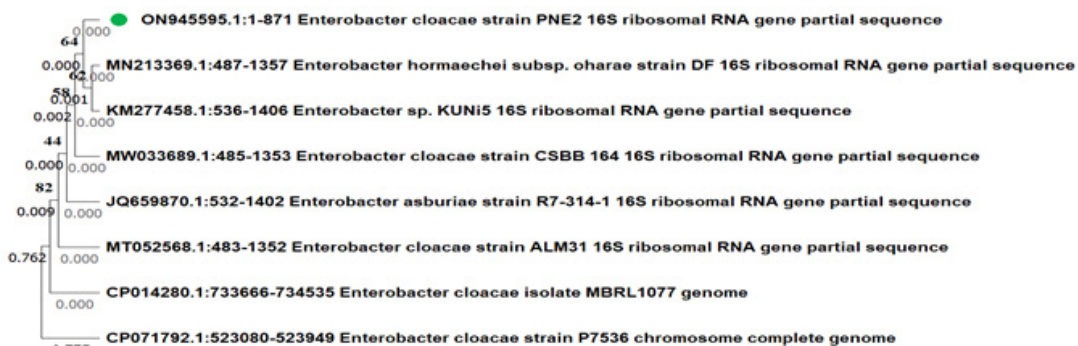
	C1	C2	T1
% Germination	58.33±2.88	66.66±5.77	73.33±5.77
Germination index (GI)	6.62±0.07	8.4±0.1	13.33±0.20
Plumule length (cm)	0.73±0.05	0.76±0.05	1±0.1
Shoot length (cm)	8.86±0.23	10.23±0.25	10.43±0.11
Root length (cm)	9.8±0.26	10.16±0.15	10.9±0.36
Vigour index (VI)	580.36	687.56	808.43
Wet weight (gm)	0.08±0.02	0.105±0.05	0.13±0.01
Dry weight (gm)	0.045±0.03	0.066±0.005	0.083±0.002

Mean ± SD = Mean + standard deviation

(C1): Unprimed control, (C2): Primed control T1: *Enterobacter cloacae* PNE2



**Fig. 1.** Cultural and morphological characteristics of the isolate *Enterobacter cloacae* PNE2



**Fig. 2.** Phylogenetic Tree of *Enterobacter cloacae* PNE2

D- arabinose, citrate utilization, and malonate utilization, three were shown negative results i.e. lactose, adinitol, rhamnose<sup>30</sup>.

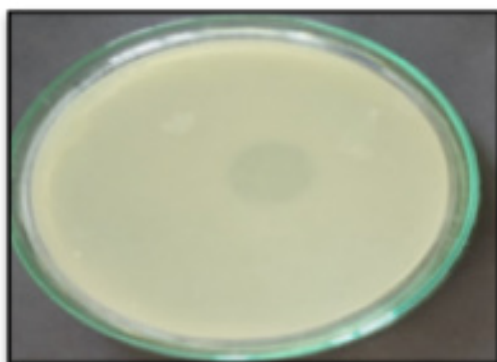
#### Molecular identification of the PNE2

Molecular identification and 16S rRNA partial gene sequence analysis confirmed that

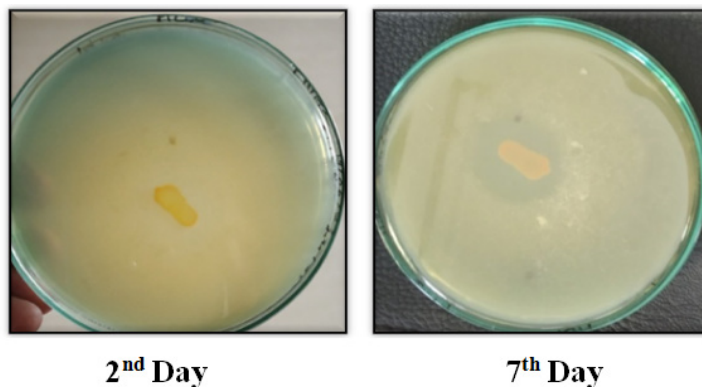
the isolate is *Enterobacter cloacae* PNE2. The phylogenetic tree for the same is depicted in figure 2. The partial 16S rRNA gene sequence of *Enterobacter cloacae* PNE2 was submitted at the NCBI Gene bank with Accession number ON945595.

#### Antibiotic Susceptibility test

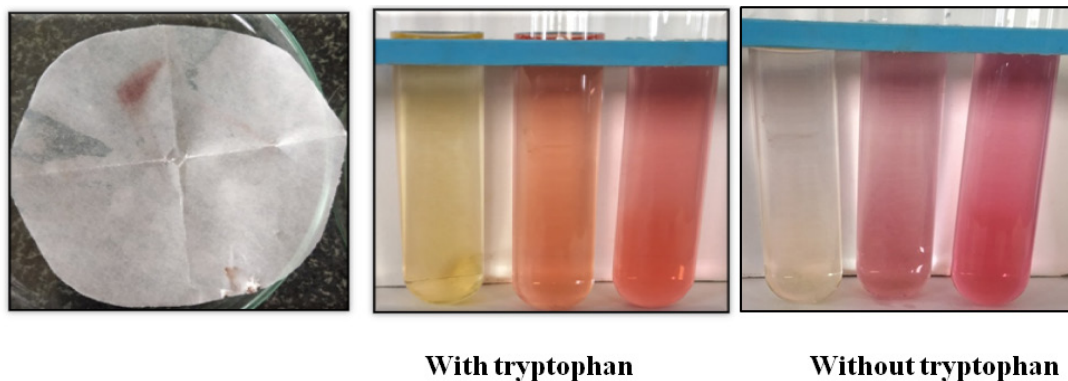
The isolate *Enterobacter cloacae* PNE2 was shown to be extremely sensitive to 22 antibiotics tested, as shown in Table 2. Compared to all 22 antibiotics tested, the isolate was most susceptible to two broad-spectrum antibiotics Carbenicillin (CB) (22 mm) and tobramycin (22 mm), followed by Ofloxacin (21). Antibiotics that inhibit protein synthesis (Tobramycin, Streptomycin, Gentamicin, Netllin, Tetracycline, Amikacin, and Kanamycin), as well as antibiotics that inhibit cell wall synthesis (Carbenicillin, Ceftriaxone, Ceftazidime, Cefepime, Cephalothin, Cefotaxime, Imipenem, Meropenem)<sup>31</sup>, are more effective against the isolate *Enterobacter cloacae* PNE2.



**Fig. 3.** Zone of Phosphate solubilization by *Enterobacter cloacae* PNE2



**Fig. 4.** Zone of potassium solubilization by *Enterobacter cloacae* PNE2



**Fig. 5.** Indole acetic acid production by *Enterobacter cloacae* PNE2



**Plant growth promoting characterization by the isolate *Enterobacter cloacae* PNE2**

**Determination of nitrite nitrogen**

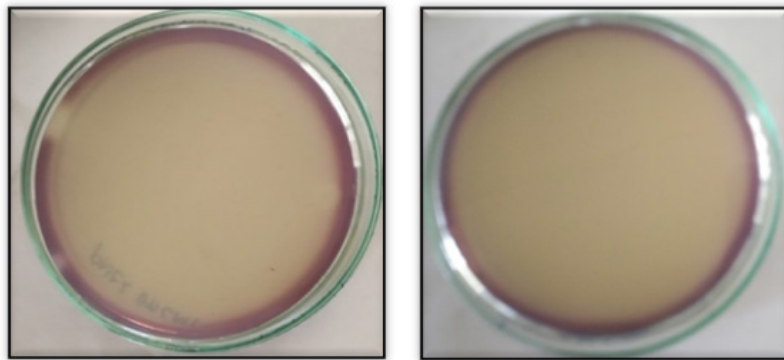
The isolate *Enterobacter cloacae* PNE2 was found to grow well in N-free Ashby's Mannitol media indicating that the isolate PNE2 was able to grow in nitrogen deficiency in media. The results in Table 3 show that isolated *Enterobacter cloacae* PNE2 produced  $0.15 \pm 0.01 \mu\text{g/ml}$  nitrite after the 8<sup>th</sup> day of incubation. The isolate *Klebsiella pneumoniae* PNE1 produced  $0.09 \mu\text{g/ml}$  nitrite after the 8<sup>th</sup> day of incubation<sup>14</sup>.

**Phosphate Solubilization activity**

The isolate *Enterobacter cloacae* PNE2

produced a clear halo zone around its colony on Pikovskaya's medium (figure 4). The phosphate solubilization index was recorded  $3.58 \pm 0.08$  after 5<sup>th</sup> day of incubation. 3.22 Phosphate Solubilization Index (PSI) was found by the isolate *Enterobacter cloacae* BAU3 after 10<sup>th</sup> days of incubation<sup>10</sup>. The Phosphate Solubilization Index (PSI) of isolate *Enterobacter cloacae* PSB6 was  $3.06$ <sup>11</sup>.

Phosphate solubilization by the isolate and change in medium pH was shown in graph 1. The range of inorganic phosphate solubilization varied from  $137.3 \pm 0.63$  to  $278.34 \pm 0.56 \mu\text{g/ml}$ . The highest solubilization of 55.66% ( $278.34 \pm 0.56 \mu\text{g/ml}$ ) was achieved on the 5<sup>th</sup> day of incubation by



**Fig. 6.** Zone of biopolymer hydrolysis by bacterial isolate *Enterobacter cloacae* PNE2



**Fig. 7.** Effects of *Enterobacter cloacae* PNE2 on *Trigonella foenum-graceum* (fenugreek) plant up to 7<sup>th</sup> days: A. (C2): Primed control, B. (C1): Unprimed control C. T1: *Enterobacter cloacae* PNE2

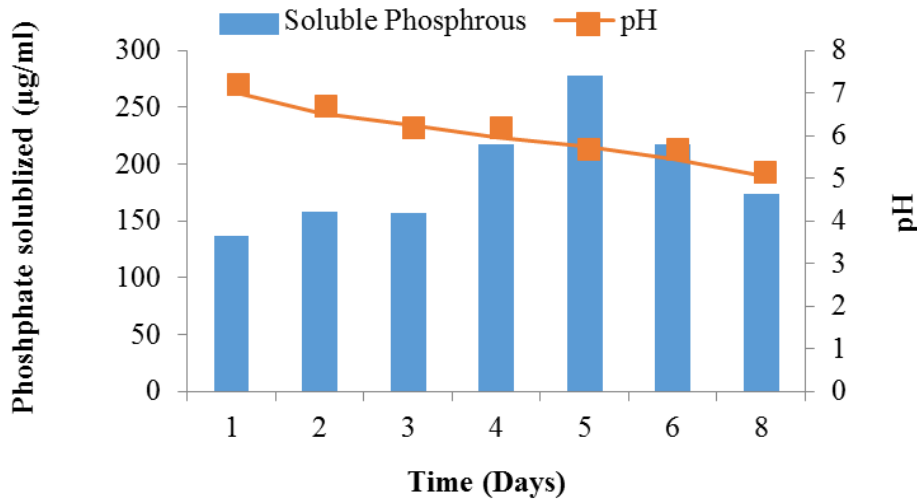
the isolate *Enterobacter cloacae* PNE2. The isolate *Enterobacter cloacae* PSB6 solubilized 96.86 µg/ml phosphorus after the 5<sup>th</sup> day of incubation, and also drop the pH from 6.90 to 4.90 after the 5<sup>th</sup> of incubation<sup>11</sup>. *Enterobacter cloacae* NII-0931 solubilized 58.5±2 µg/ml phosphorus after the 15<sup>th</sup> day of incubation<sup>32</sup>. Both strain *Enterobacter cloacae* PSB6 and *Enterobacter cloacae* NII-0931 are low amounts of phosphate solubilization as compared to *Enterobacter cloacae* PNE2.

**Determination of potassium solubilization activity**

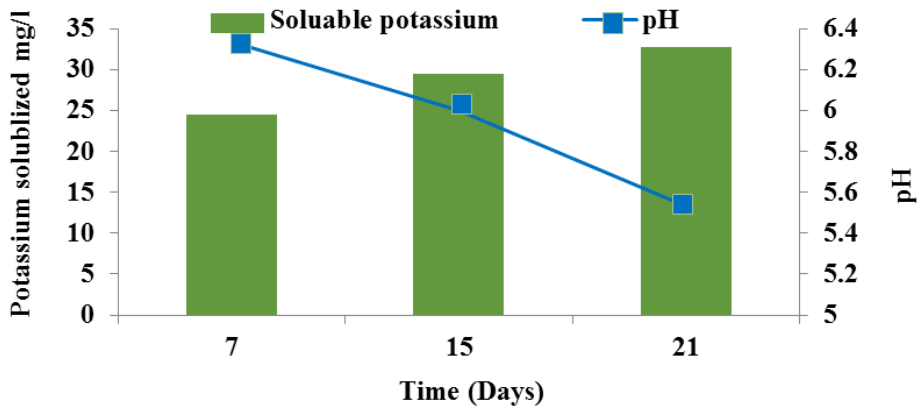
Table 4 showed the Potassium solubilization index of isolate *Enterobacter cloacae* PNE2 varies in ranges from 1.6 to 2.18 on Aleksandrov medium with bromothymol blue

within 7<sup>th</sup> days of incubation. The isolate showed yellow color formation around the growth in the Aleksandrov medium with bromothymol blue its indicating that the isolate might be produced organic acid to solubilize the potassium from the medium (figure 4). The potassium solubilizing index by the isolate *Enterobacter cloacae* PSB6 was 3.05<sup>11</sup>.

The quantitative estimation amount of Potassium released from feldspar revealed the Potassium solubilization ability of the isolate *Enterobacter cloacae* PNE2 (graph 2). The range of inorganic Potassium solubilization varied from 24.53±0.06 mg/l to 32.69±0.39 mg/l. The maximum solubilization of 32.66 mg/l was achieved on the 21<sup>st</sup> day of incubation by the isolate *Enterobacter*



Graph 1. Quantitative estimation of soluble Phosphorus by *Enterobacter cloacae* PNE2



Graph 2. Potassium solubilization potential of isolate *Enterobacter cloacae* PNE2

*cloacae* PNE2. The isolate *Enterobacter cloacae* PSB6 solubilized 14.00 mg/l of potassium on the 5<sup>th</sup> day of incubation<sup>11</sup>. The isolate *Enterobacter cloacae* GL7 solubilized less than 2 mg/l potassium<sup>12</sup>. *Enterobacter cloacae* 38 solubilized 71.15 µg/ml potassium after the 5<sup>th</sup> day of incubation<sup>33</sup>.

#### Indole Acetic Acid (IAA) production

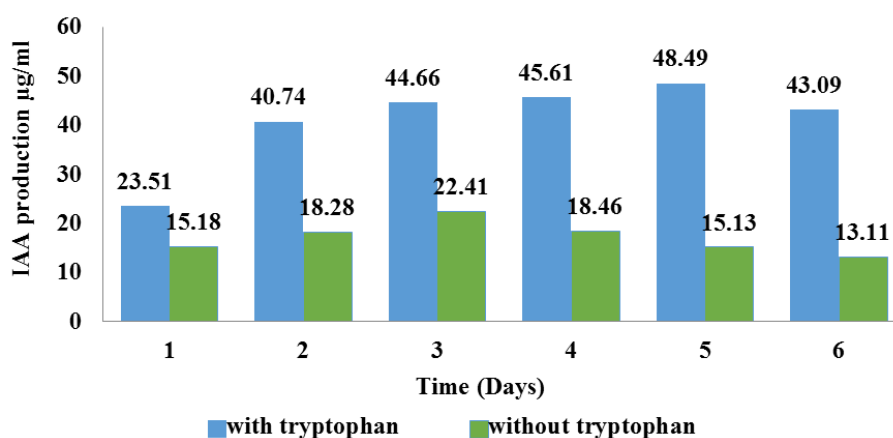
The qualitative determination of IAA production by the isolate *Enterobacter* PNE2 was shown in Figure 5. The isolate formed pink color on the filter paper after 72 hrs incubation, which confirmed that the isolate can produce IAA.

As shown in graph 3, *Enterobacter cloacae* PNE2 produced a substantial amount of IAA both in the absence and presence of tryptophan. In presence of tryptophan, IAA production ranges from 23.5±0.45 to 48.49±0.05 µg/ml. Maximum IAA production was recorded 48.49±0.05 µg/ml in presence of tryptophan after 5<sup>th</sup> day of incubation.

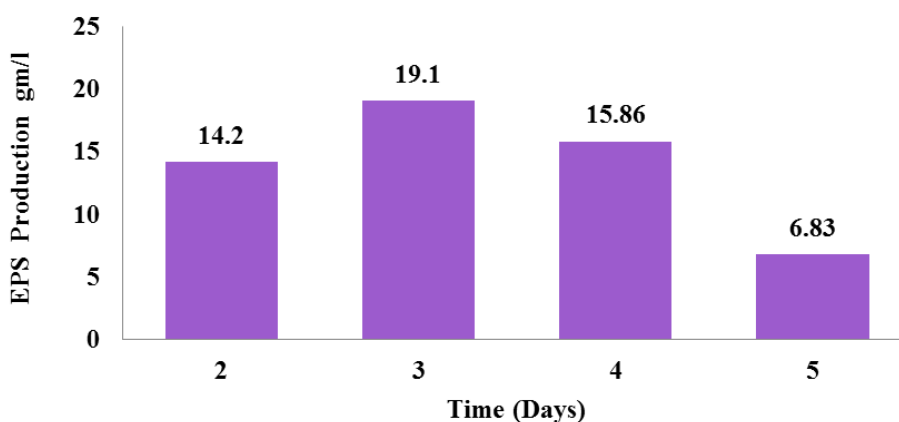
In absence of tryptophan, IAA production ranges from 15.18±0.03 to 22.41±0.01 µg/ml. Maximum IAA production was recorded 22.41±0.01 µg/ml in absence of tryptophan after 3<sup>rd</sup> day of incubation. *Enterobacter cloacae* PNE2 showed maximum IAA producing ability in presence of tryptophan indicating that it might be the precursor for IAA biosynthesis. The isolate *E. cloacae* JAS7 produced 46.6±0.5 µg/ml IAA<sup>34</sup>. The isolate *Enterobacter cloacae* produced 2.443 µg/ml of IAA<sup>35</sup>. The isolate *Enterobacter cloacae* H3 produced 12.28 µg/ml IAA after 5<sup>th</sup> day of incubation<sup>13</sup>.

#### Quantitative estimation of EPS production

Data presented in graph 4 depicts EPS Production by *Enterobacter cloacae* PNE2 using 5% sucrose as substrate. The amount of EPS produced ranged from 6.83±0.11 to 19.1±0.2 g/l. The EPS production increased till the third day and thereafter it continuously decreased. Thus,



Graph 3. Quantitative estimation of IAA production by *Enterobacter cloacae* PNE2



Graph 4. EPS Production by *Enterobacter cloacae* PNE2 using 5% sucrose

maximum production could have occurred around the 3<sup>rd</sup> day. The decrease in the number of EPS after the 4<sup>th</sup> day may be due to the utilization of own EPS as substrate by the producing organism or EPS yield may probably decrease because of the action of glycohydrolases produced in the culture that catalyzed the degradation of polysaccharides, resulting in decreased EPS yields<sup>37</sup>. After the fourth day, EPS production may have been reduced because the isolate began to utilize its own EPS as a substrate. EPS yield may also have dropped as a result of the activity of glycohydrolases created in the culture, which catalyzed the breakdown of polysaccharides<sup>37, 38</sup> reported 8.83 g/l of EPS yield of the isolate *Enterobacter cloacae*.<sup>15</sup> reported 12.95 g/L of EPS yield by *E. cloacae* Z0206.<sup>36</sup> reported 18.1 g/l of EPS yield by *Enterobacter sp.* strain ACD 2 after the 3<sup>rd</sup> day of incubation

#### **Determination of Potential for biopolymer degradation**

As shown in Table 5, the isolate *Enterobacter cloacae* PNE2 could degrade cellulose, pectin, and xylan. Based on the zone ratio the ability of the isolate to degrade biopolymers was best for pectin, good for cellulose, and least for xylan. The zone ratio for cellulose degradation was 5.5 mm. The zone ratio for Pectin degradation was 9.2 mm and the zone ratio for Xylan degradation was 1.6 mm. The isolate *Klebsiella pneumoniae* PNE1 had a good ability to degrade cellulose, pectin, and xylan<sup>14</sup>.

#### **Seed germination assay**

Figure: 7 showed the effects of *Enterobacter cloacae* PNE2 on *Trigonella foenum graecum* (fenugreek). Table 6 showed the seed germination and seedling growth of *Trigonella foenum graecum* (fenugreek) plant till 7<sup>th</sup> day of observation. When the *Trigonella foenum graecum* (fenugreek) seed was treated with the plant growth promoting isolate *Enterobacter cloacae* PNE2, significantly improved ( $P < 0.05$ ) in growth of *Trigonella foenum graecum* (fenugreek) plant was observed as compared to unprimed control in terms percentage germination (73.33±5.77), germination index (13.33±0.20), plumules length (1±0.1), shoot length (10.43±0.11), vigour index (808.43), wet weight (0.13±0.01), dry weight (0.083±0.002) and root length and also compare to primed control in terms germination index (13.33±0.20), plumules length (1±0.1), root length, vigour index (808.43),

wet weight (0.13±0.01), dry weight (0.083±0.002) and root length. No significant difference was found between primed control (C2) and T1 (*Enterobacter cloacae* PNE2) in terms of percentage germination, shoot length and dry weight.<sup>11</sup> reported that the isolate *Enterobacter cloacae* use in seedling growth of fennel (*Foeniculum vulgare* Mill) crop.<sup>33</sup> reported *Enterobacter* spp. significantly improved overall germination parameters and vigour index of okra seed.

### **CONCLUSION**

Based on morphological, biochemical, and 16S rRNA partial gene sequencing analysis, the isolate obtained from vegetable market waste has been identified as *Enterobacter cloacae* PNE2. The antibiotic susceptibility test indicated that the isolate was found to be sensitive to all 22 antibiotics tested. Plant growth promoting characterization revealed that the isolate was capable of producing nitrite and solubilizing phosphate and potassium through the biological process from unavailable to readily available forms. The ability of the isolate *Enterobacter cloacae* PNE2 to produce nitrite and solubilized potassium and phosphorous will enhance the nutrient status of soil as these are essential for the growth of the plants. Furthermore, the isolate *Enterobacter cloacae* PNE2 can also produce considerable amounts of phytohormones (Indole acetic acid) and exopolysaccharides (EPS), which will enhance survival and promote plant growth. Moreover the isolate *Enterobacter cloacae* PNE2 was also found capable of degrading biopolymers viz., cellulose, pectin, and xylan. The isolate *Enterobacter cloacae* PNE2 to enhance the seedling growth of *Trigonella foenum graecum* (fenugreek) plant as compared to the control. Thus, the current study demonstrates that the isolates have a variety of plant growth promoting traits, which are beneficial to crops when employed as biofertilizers in agricultural fields.

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#### Conflict of interest

The authors would hereby like to declare that there is no conflict of interest.

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