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STUDY OF VARYING pH RANGES ON THE GROWTH RATE OF BACTERIAL STRAINS ISOLATED FROM PLANTS

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The effect of different pH ranges 3-10 was studied on the growth rate of six different bacterial strains, e.g. *Escherichia coli, Klebsiella Pneumoniae, Bacillus cereus, Bacillus flexus, Brevibacterium borstelensis, Staphyllococcus scuiri*, isolated from different hosts (Kinnow, Gada Dahi, Sweet orange, Musambi, and Bindweed).These bacterial endophytes were distinguished by the use of universal 16s rRNA primers based on morphological characters and biochemical tests as well as molecular approaches.The amplified PCR products were purified and sequenced from Mobix at Mc Master's University, Hamilton, Canada, and submitted to the NCBI database under accession (LT844655, MF966247, LT745986, LT745989, LT745979, and MF977365). The phylogenetic tree of isolated strains was constructed by MEGA 7.0 software. Gen 5 plate reader software was used to measure growth at O.D 600nm in 96 well plate for 24 hours at 30^{o}C in EpochTM microplate spectrophotometer. According to the findings, *Escherichia coli* grew best in the pH ranges of 6, 7, and 8, but not in the pH range of 9. Similarly, *Staphylococcus scuiri* grew best in a pH range of 7 to 10, while pH 4 and 5 had no development.The typical growth pattern of *Klebsiella pneumoniae* was observed at pH 9 and 10, whereas growth was slower or limited in the other pH ranges. Whereas, *Bacillus flexus* and *Bacillus cereus* grew best at pH 6-9. *Brevibacterium borstelensis*, on the other hand, grew best at 4-9.These findings indicate that pH can play an important role in determining bacterial competition in host plants.

Keywords: Growth rate, bacterial endophytes, pH ranges,16SrRNA.

INTRODUCTION

Since pH is a logarithmic feature, a one-pH shift corresponds to a ten-fold change in hydrogen ion (H+) concentration (Pocock *et al*., 2018).The pH is the most effective element for microbial growth and has a significant effect on cells, typically with growth in the range of 2-3 pH units and close to neutral 7.0 pH. Microbes released certain acid or alkali metabolites in the medium during their growth.The pH in both external and internal systems must be similar to neutral for microbial cells to expand normally. pH can affect the nature of the proteins that determine the rate of bacterial growth. pH variations in the external environment also modify the ionization of nutrient molecules and reduce their availability to microbial cells, thus inhibiting microbial growth.The microbial diversity in the soil is predicted by soil pH and nitrogen-based fertilizers, according to research (Fierer and Jackson, 2006; Rousk *et al*., 2010; Lauber *et al*., 2008; Lauber *et al*., 2009). Microbial diversity of soil was shown to be higher in neutral soils and lower in acidic soils (Rousk *et al*., 2010).

Roesch *et al*. (2007) found that forest soil with low pH had more variation than agricultural soil with high pH. Bacterial strains have certain limits to the tolerance of acidity when the pH of the external environment of the cell is lower than internal at that point when the H^+ moves towards the cytoplasm to lower the internal pH of the cell.This difference in the pH of the microbial cell may be lethal to bacterial growth, often some prokaryotes have died when the internal pH decreases below 5.0-5.5, which can destroy the plasma membrane and may impede the action of the enzyme or some membrane transport proteins (Madigan *et al*., 2000). pH also has a significant effect on the composition of the microbial community in soil or other ecosystems (Jones *et al*., 2009; Ratzke *et al*., 2018). Different biochemical processes, on the other hand, require proton turnover, and microbes may also alter the pH of the surrounding environment.Microbes have an optimum pH range of 7, 8 that varies by species and atmosphere. pH above or below this optimum range could inhibit or could inhibit or even can cause death. The ways how microbes modify pH also influence the growth of other microorganisms. This determined behavior and interaction between bacterial species based on the pH change of the environment.

In this research, the pH influence was studied as a major factor for the survival of the soil microbial community as beneficial bacterial endophytes could be applied to crops to improve their growth and productivity. Bacterial endophytes

seem to be interesting not only because of their beneficial impact on plant growth but also because they are a safer and environmentally friendly alternative to fertilizers. As a result, it is important to determine the pH at which certain bacterial strains could survive.The main objective of this work was to see how the pH spectrum (3-10) influenced the growth rate of six tested bacterial strains and which pH range is suitable for their proper growth and development.

MATERIALS AND METHODS

During the year 2016, a total of 25 samples of Kinnow (5), Gadadahi (5), Sweet orange (5), Musambi (5), and Bindweed (5) were collected from various locations across Punjab, including Lahore, Sargodha, Faisalabad, and Mian Chanu.

***Isolation of bacterial cultures*:** Isolation of bacteria was performed by grinding the midrib portions of leaf samples in sterile distilled water under sterile conditions and the filtrate was spread on the LBA medium plate and incubated at 37ºC for 24 hours.The next day, bacterial colonies that had appeared on the media were purified by streaking morphologically distinct colonies on LBA plates and incubating for another 24 hours at 37ºC. Subsequently, morphologically diverse strains continued with morphological, biochemical characterizations, accompanied by the Burgey Manual of Formal Bacteriology and Molecular marker 16sRNA as mentioned below.

***DNA Isolation from Bacterial Culture*:** Genomic DNA of six bacteria was isolated through the CTAB method described by (Wilson, 2001). The bacterial culture was grown in 3mL of growth medium (LB) for 24 hours and centrifuged at 13000 rpm for 2 minutes. For the cell lysis, the pallet was dissolved in 567µL of TE buffer and 30µL of 10% SDS was added. After that, 3L of proteinase k (20 mg/mL) was added and incubated for 1 hour at 37°C. The suspension of bacterial cells was then diluted with (100L of 5M NaCl + 80L of CTAB) and incubated for 10 minutes at 65ºC. The upper aqueous phase was then moved to a new tube after centrifuging 500L of Chloroform and Iso-amyl alcohol for 5-10 minutes. After that, 20L of 3M Sodium Acetate and 500L of Absolute Ethanol (100%) were applied and gently mixed to precipitate DNA before being stored at -20ºC overnight. The tubes were centrifuged at 13000 rpm for 10 minutes the next day, and the supernatant was discarded. The pallet was resuspended in 50L of TAE buffer after being washed with 70% ethanol. The results were checked for 1% (w/v) agarose gel stained with ethidium bromide.

***PCR amplification of DNA*:** For amplification of DNA by PCR a reaction mixture of 25 µL were prepared by mixing 0.12µL 10X Taq polymerase buffer, 4µL mM $MgCl_2$, 1µL mM dNTPs, 1µl of 10 pmol forward primer 27-F (5' AGAGTTTGATCMTGGCTCAG 3'), 1µl of 10 pmol reverse primers 1492-R (5´ ACCTTGTTACGACTT 3´), 3µL DNA, d.H_2O 9.88 µl and 0.12 µl Taq DNA polymerase (Fermentas) in a PCR tube (0.25 µL). A thermal cycler was used to incubate the reaction mixture (Eppendorf). A preheating procedure was accompanied by 32 cycles of 94°C for 5 minutes, 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by a final incubation of 10 minutes at 72°C.

***Gel extraction*:** The PCR amplified DNA was run on a 1% agarose gel (stained with ethidium bromide) and the desired fragments were removed from the gel using a sharp Blade on a UV transilluminator system. Amplified PCR product was extracted from the gel piece by using Gene All kit followed by manufacturer instructions.

Sequencing: Purified PCR products were sent to Macrogen in Korea, where they were sequenced using an ABI PRISM Big Dye Terminator cycle sequencer with M13F and M13R primers. The 16S rRNA gene sequences obtained were matched by aligning the results with the Gene Bank sequences using the Basic Local Alignment Search Tool (BLAST) search tool at the National Center for Biotechnology Information (NCBI).

***Phylogenetic analysis*:** To determine the exact nomenclature of the isolates, the sequencing results have been blast-off by NCBI, and sequences of related species have been collected. Phylogenetic analysis was performed using MEGA-7 bioinformatics software to determine the evolutionary pattern among known bacterial strains.

***Growth curve experiment*:** Bacterial isolates were inoculated in 5mL LB broth (without antibiotics) and incubated for 24 hours at 30ºC. Cells that had been cultured overnight were harvested. 1.5 mL of culture transfer was transferred to a new 1.5 mL tube, centrifuged at 13,000 rpm for 1 minute, and the procedure was repeated until all cells were harvested. 1mL of LB broth was used to suspend the bacterial pellet. A spectrophotometer was used to calculate the culture's optical density at 600nm. 1mL of LB broth was used in a 1.5mL cuvette for the blank. After that, the optical density (O.D.) of all samples was calculated at 600nm and adjusted to 1.5, and the LB broth media with different pH values were prepared and autoclaved. According to the experimental plan, each sample was mounted onto a 96-well plate as follows. In columns 1, 3, 5, 7, 9, 11, load 15 µL of bacterial culture and 135 µL of LB media filled with various pH ranges as pH 7 was considered as control. In columns 2, 4,6,8,10,12, add 150 µL of uncultivated LB media which served as control for bacterial infection. 96 well plate was properly sealed with a tap to prevent moisture from leaking out of the plate during growth curve development. Gen5 Plate Reader app was opened and a 24-hour growth curve was picked in the Experiments folder, and this growth experiment was shaking (slowly) at 30ºC and calculated O.D 600nm.The plate was put in the unit and the start button was pressed. Once the 24-hour protocol was completed, the

reading was saved in an Excel file for further testing and the formation of bacterial growth curves.

RESULTS AND DISCUSSION

These test bacterial cultures were isolated from different varieties of citrus (Kinnow, Gada Dahi, Sweet orange, Musambi) and weed (Bindweed) from different locations of Punjab, and six different genera of bacteria were selected for this study as described in (Table 3). These distinct strains of bacteria were identified through morphological, biochemical traits as stated in Bergey's Systematic Bacteriology Manual (George, 2005) and 16SrRNA (Table 1,2,3).

Biochemical tests for isolated bacterial strains are listed in (Table 2) and three strains of *Klebsiella pneumoniae*, B. cereus, E. coli were gram-negative, while others (*Brevibacillus borstelensis, B. flexus, Staphylococcus scuiri*) were gram-positive. All five strains were rod-shaped except for *Staphylococcus scuiri* (cocci). Some strains were non-motile, such as *Klebsiella* and *Staphylococcus*, while others were motile (Salo *et al*., 2020).

Sequenced samples were analyzed using DNA star software and blast in the national database for the identification of bacterial strains. Based on the findings *Bacillus cereus, B. flexus, Brevibacillus borstelensis, Staphylococcus scuiri, Klebsiella pneumonia*, E.coli were classified and these strains belong to phylum Firmicutes and Proteobacteria. All identified strains were shown percent identity in NCBI (93-

Table 1. Morphological characterization of isolated bacterial endophytes from leaves of citrus Varieties and weed

Isolate	Bacterial Isolate	Colony color	Colony texture	Shape	Margins	Elevations
SM-42	*Brevibacillus borstelensis*	White	Smooth and rough crust	Round	Irregular	concave
SM-25	*Bacillus cereus*	Dirty off white	Smooth	Round	Irregular	flat
SM-WD23	*Bacillus flexus*	Off white	Smooth and shiny	Round	Irregular	Slightly raised
SM-8	*Staphylococcus Scuiri*	Dirty off white	Smooth and shiny	Round	Irregular	Flat
SM-82	*Klebsiella pneumoniae*	Dirty white	Smooth	Round	Wavy	Raised
SM-WD6	*Escherichia coli*	Dirty off-white	Smooth	Round	Slightly wavy	Raised

Note: WD stands for bacterial strains isolated from the weed as host

Table 2. Biochemical characterization of isolated bacterial endophytes from leaves of citrus varieties and weed

Bacterial	Biochemical Tests														
	Cell shape	Gram type	Spore type	Capsule stain	Motility test	Indole test	Methyl red test	Citrate utilization test	Hydrogen sulphide test	Nitrate reduction test	Oxidase test	Catalase test	Growth at 2% NaCl	Growth at 25°C	Growth at 40 °C
Brevibacillus borstelensis	rod	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
B. cereus	rods	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve
Bacillus flexus	rods	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
Escherichia coli	rod	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve
Klebsiella pneumoniae	rod	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Staphylococcus scuiri	cocci	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve

Note: +ve = positive, –ve = negative

Table 3.Details of characterization of isolated endophytic bacterial strain from citrus varieties and weeds based on 16sRNA analysis.

Isolate	Bacterial isolate	Phylum	Host	Scientific names of host	Location	NCBI Accession no.	Percent identity
SM-25	*Bacillus cereus*	Firmicutes	Kinnow	*Citrus reticulate*	Faisalabad	LT844655	99%
SM-82	*Klebsiella pneumoniae*	Proteobacteria	Gada dahi	*Citrus aurantium*	Sargodha	MF966247	98%
SM-WD6	*Escherichia coli*	Proteobacteria	Bind weed	*Convolvulus arvensis*	Lahore	LT745986	96%
SM-42	*Brevibacillusborstelensis*	Firmicutes	Sweet orange	*Citrus sinensis*	Mian Chanu	LT745989	93%
SM-WD23	*Bacillus flexus*	Firmicutes	Bindweed	*Convolvulus arvensis*	Lahore	LT745979	99%
SM-8	*Staphylococcus scuiri*	Firmicutes	Musambi	*Citrus sinensis*	Sargodha	MF977365	99%

100%) with other reported strains. These sequences were submitted to NCBI with accession no. (LT844655, MF966247, LT745986, LT745989, LT745979 and MF977365) as described in (Table 3). The phylogenetic tree was created with MEGA 7 software using the muscle and neighbor-joining algorithm as presented in (Figure 1). Major two clades were formed in a tree which divides two strains *Klebsiella sp*. and *E. coli* into separate clades and these two strains were genetically more similar with each other as lies in neighboring clades as compared to others. The second clade was further subdivided into four branches, with Brevibacillus and Staphylococcus sharing the closest branches and Bacillus strains clustered together. The bacterium Cyanobacteria was used as an outgroup in this study, and it belongs to a distinct clade below the main two, indicating that it has no genetic resemblance to the studied ones.

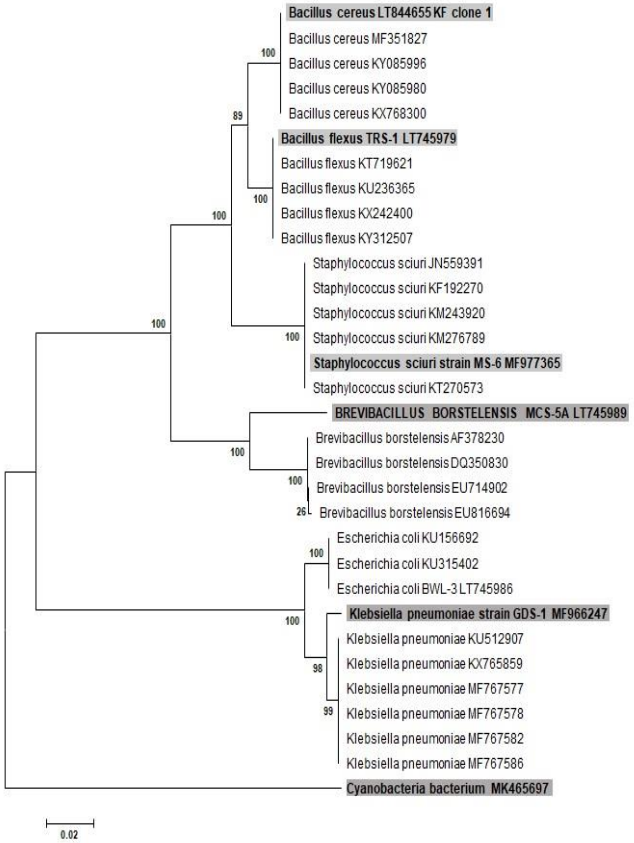

Figure 1. Phylogenetic dendrogram of isolated bacterial strain constructed by using Neighbor-Joining algorithm in MEGA 7. Total thirty-two sequences were used to construct the neighbor-joining phylogenetic tree and among them on the sequence of *Cyanobacteria bacterium* was used as an outgroup.

The evolution of microbes has long been influenced by environmental factors. Each microbe has a particular growth requirement, that's why complex environmental conditions determine the diversity of microbial populations and their interactions in soil and among plants. pH is a very important element for microorganisms, and each microbe needs a specific pH spectrum to grow and reproduce in its hosts. Indeed, pH is believed to be the major key factor behind the normal occurrence and interaction of microbes in many ways, such as intestinal microbes (Sofi *et al*., 2013), oral (Bowden, 1987; Quivey, 2000), and plant-associated microbes (Rashid *et al*., 2012).

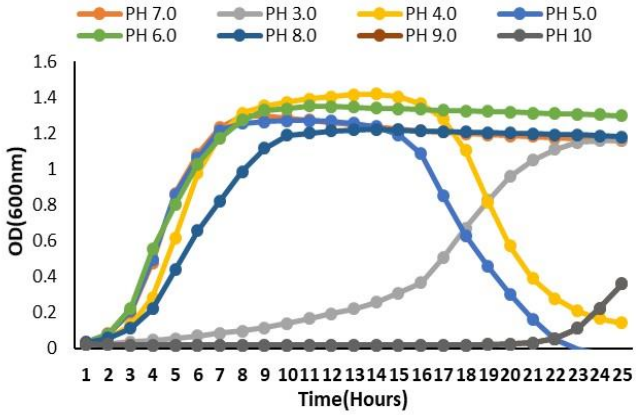

Figure 2. Effect of different ranges of pH on the growth of *Escherichia coli* at 30ºC for 24 hours at OD (600nm).

***Effect of pH on bacterial growth*:** The data for the growth curve of *Escherichia coli* in different pH ranges for 24 hours at O.D (600nm) is presented in (Figure2). According to the findings, Escherichia coli grew more at a neutral pH of (7), whereas it grew less at a pH of 3 until 16 hours, after which it grew well for the next 24 hours. In the case of pH 4 and 5, there was more growth before 17 hours, after which the rate of growth slowed from 17 to 24 hours. Maximum growth was observed at pH range (6, 7, 8) while growth was suppressed at pH 9, 10 to 21 hours, followed by a slight increase after 21 hours at a temperature of 30ºC.

The effect of different pH ranges on Bacillus cereus growth is shown in (Figure 3). The growth curve indicated that B.cereus shows maximal growth on all pH ranges, except pH 3.0, where it dropped after 19 hours. At pH 10, B. cereus did not show any growth until 17 hours, where it abruptly began to rise.

The turbidity of the culture medium, as well as the length of the lag period, growth rate at logarithmic phase, and a maximum population that the medium can sustain, can all be used to estimate bacterial growth. This research was designed to assess the impact of pH variations on all of the test microbes as well as to determine the optimal pH for each strain. Hinshelwood (1946) investigated the impact of pH on

the growth of *Bac.zactis aerogenes* at the population level and found that no growth was restricted during the lag phase and that the rate of growth was normal. Although the pH have an impact to predict the growth of various bacterial species isolated from alkaline soils (Sovljanski *et al*., 2019). As a result of the study, it was concluded that the maximum population of bacteria in the medium could not be stable unless nutrients were provided, and these findings contradicted proof of the maximum population in both acidic and alkaline environments.As a result, optimum pH is needed for maintaining a constant rate of cell growth and viability.

The effect of different pH growth ranges on *Brevibacterium borstelensis* growth is shown in (Figure 4). This bacterium grew normally in all pH ranges, except pH 3.0, where it did not grow for 16 hours before reaching its maximum growth rate.

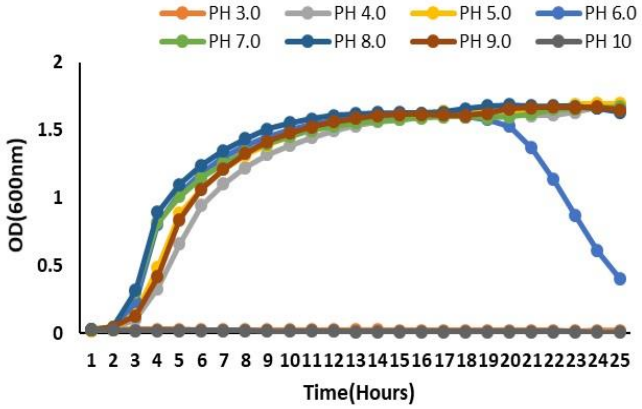

Figure 3. Effect of different ranges of pH on the growth of *Bacillus cereus* at 30ºC for 24 hours at OD (600nm).

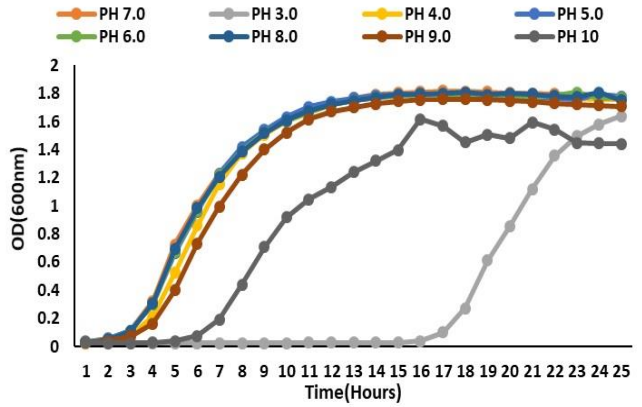

Figure 4. Effect of different ranges of pH on the growth of *Brevibacterium*halotolerans at 30ºC for 24 hours at OD (600nm).

Shimwell (1935) used the *Lactobacitlus pastorianus* strain and found that as the pH was decreased as a measure of growth, the lag phase increased. However, it is clear that the pH had an impact on the bacterial culture during a single period of development, and that this effect varied between microbial species and culturing methods.

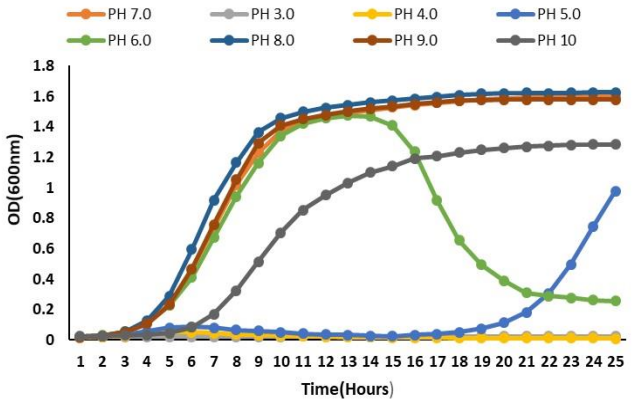

Figure 5. Effect of different ranges of pH on the growth of *Staphylococcus scuiri* at 30ºC for 24 hours at OD (600nm).

After twenty-four hours at 30^0C, the effect of various pH ranges on the growth rate of *Staphylococcus scuiri* was investigated at OD (600nm). At pH 4, no growth was observed, while at pH 5, growth was observed after 19 hours, and at pH 6, the growth rate declined nearly 15 hours before linearizing after 22 hours. This bacterium grew normally in other pH ranges of 3, 7, 8, 9, and 10 (Figure 5).

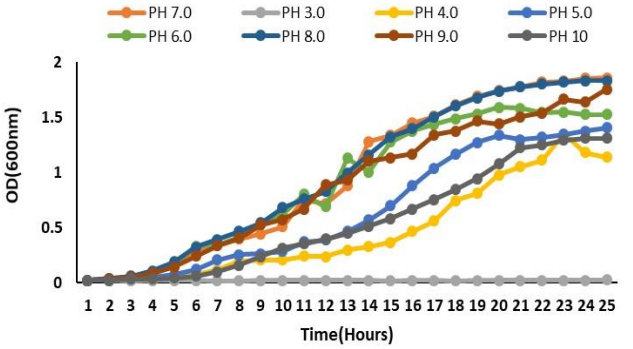

Figure 6. Effect of different ranges of pH on the growth of *Bacillus flexus* at 30ºC for 24 hours at O.D (600nm).

Development curves for *Bacillus flexus* demonstrated normal potential for all pH ranges (Figure 6). There were strong peaks observed at pH 5,6,7,8, and 9. Other pH ranges, on the other hand, showed a natural growth rate.

Gale and Epps (1942) demonstrated that the behavior is striking with the sensitivity for the acidity of enzymes of *Bact. cozi* sensitivity in contrast to acidity. Change of pH affects the efficiency of the growth rate of cells in the

specific medium at a specific temperature. However, the cells typically perform better at the lower end of the pH spectrum than at the higher end, implying that pH is a significant factor in growth. Jordan and Jacobs (1947) found that at pH 7, the ratio of total viable cells was similar to the initial phase, which was roughly equivalent to pH 2.0 over a wide temperature range.The growth curve of *Klebsiella pneumoniae* showed a normal pattern at pH 9, 10, despite the fact that the bacterium's growth rate slows after 15 hours and curves decline in the rest of the pH ranges (Figure 7).

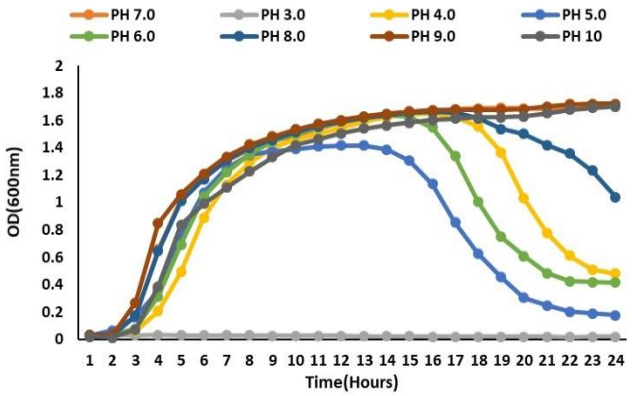

Figure 7. Effect of different ranges of pH on the growth of *Klebsiella pneumoniae* at 30ºC for 24 hours at OD (600nm).

In this research, it was observed that Escherichia coli grew best at pH levels of 6, 7, and 8, but not at pH 9. Similarly, in the case of *Staphylococcus scuiri*, the best growth was 7-10 pH, although no growth was observed in pH 4 and 5. In the case of *Klebsiella pneumoniae*, a natural growth pattern was observed at pH 9 and 10, while no growth was observed in the rest of the pH. *B. flexus* and *B. cereus* showed maximum growth at pH 6-9. However, *Brevibacterium borstelensis* gave maximum growth at 4-9, but each strain gives the best growth at pH 7 and starts to grow at pH 2.0. There is very little literature available, particularly on these bacterial strains. This study is new, no one optimizes the pH of the bacterial strains and brings novelty to this work. Since studying the impact of endophytes on plants and then their effects on the growth rate of these bacteria was studied. Through extensive research and testing on plants, it was found that these endophytes have the potential to promote plant development, and hence are known as plant growth promotors. We tested the endophytes' plant growth-promoting properties on six different vegetables and citrus plants in previous studies (Mushtaq *et al*., 2018 a; Mushtaq *et al*., 2018 b; Mushtaq *et al*., 2019; Mushtaq *et al*.,2020).Since these bacteria are tested and found to be involved in plant growth promotion, the evidence provided in this manuscript focuses on the impact of pH on bacterial endophytes growth. As a result, the pH spectrum of these bacteria was investigated to see in which types of soils they could be used as plant growth promoters instead of fertilizers.

Conclusion: Since soil pH is such an important factor in determining the form of vegetation on a given piece of land, as well as the availability of nutrients to plants. Basic/ Alkaline pH decreases the solubility of micronutrients to plants such as (Zn, Fe, Cu, and Mn) except for chloride (Cl) and Molybdenum (Mo).The majority of nutrients are basic nutrients, such as phosphates, which are available to plants at a pH range of 6.5 to 7.5. (6.5-7.5). However, pH also influences the diversity of beneficial microbes in the soil that ultimately affects plant growth. As a result, it is concluded that *Escherichia coli, B.flexus, and B. cereus, Brevibacterium borstelensis,* grow best in the pH range of 6-8 and are ideal for use as plant growth promoters in soils to improve plant growth and productivity.

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