# Isolation of Glucose Fermenting Nitrogen Fixing Gene (nifH) Containing Endophytic Bacteria from Seed and Roots of Wheat (Triticum aestivation l.)

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**Research Article**

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# Isolation of Glucose Fermenting Nitrogen Fixing Gene (nifH) Containing Endophytic Bacteria from Seed and Roots of Wheat (*Triticum aestivation* l.)

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**Abstract**

The present study was conducted to study the *nifH* gene containing endophytic bacterial frequency in wheat seed and roots obtained from the soil of Chitwan and Kaski. One hundred and four isolates were studied for the presence of the *nifH* gene. There was a diversity in isolate characters obtained from root sample Root (R), direct seed sample (ds), and plant sample (P). None of the isolates from any sources showed indole-producing ability. About 18 isolates (15% of the total) contained the *nifH* gene through amplification of the gene by universal primers PolF and PolR. About 6 isolates from seed sample ds and 12 isolates from root sample R contained *nifH* gene. None of the isolates from root sample P manifested the presence of the *nifH* gene. Among 18 *nifH*-containing isolates, only 6 isolates manifested presence of *cel3* gene of 400 bp, whereas, 11 isolates showed *cel3* gene of 200 bp. All *nifH* gene containing isolates were confirmed to be bacteria by PCR amplification of 16s rRNA gene by universal primer 27F and 1492R and visualization of agarose gel matrix with bp range of approximately 530 under UV ray. Further research scope exists to use these microbes as a bio fertilizer in plant growth promotion studies by inoculation.

**Key words**: Citrate Utilization, Endophytic Bacteria, *nifH* gene, Plant growth promotion, Wheat

## 1 Introduction

Endophytes are microbes living inside plant region for their certain life cycle without affecting the host (Puri et al., 2018). Endophytes may become active at favorable condition and can be suppressed during host stress condition such as limited carbon source (Jia et al., 2016). Such bacteria are considered to promote plant growth through various direct mechanisms like biological nitrogen fixation, phosphate solubilization or synthesis of phytohormone (Mirza et al., 2000). Some endophytes are proven to increase plant growth by mobilizing nutrients and maintaining plant hardiness (Hardoim et al., 2008). Iniguez et al., (2004) also defined such endophytes as diazotrophs which can harbor inside plant without causing any particular harm or disease to plants and convert atmospheric nitrogen into usable for by plant system (Aguilar et al., 1998). Bacterial variations in diversified ecosystem are due to multi-layered cell wall structure, endospores and extracellular enzymes. Occurrence, distribution and prevalence of microbial communities in their niches could be influenced by factors like plant growth stage, soil pH, root exudates and climatic factors (Puri et al., 2018; Vessey, 2003). Prospects of utilizing such isolates through inoculations in organic agriculture is changes as it could replace inorganic fertilizers is also pointed by (Puri et al., 2018). Chelius and Triplett (2000) isolated isolates from maize having broad range capable of colonizing endophytes. Endophytes can pass from one generation to another via seeds, called vertical transmission (Abdelfattah et al., 2021). If nutrients like nitrogen are restricted in field, plant growth will be limited (Beneduzi et al., 2008). Agricultural inputs like chemical fertilizers generally decrease soil biomass affecting agroecosystem (Geisseler & Scow, 2015). Talking about the context of Nepal, study on leaching of nitrate from cropped rain fed terraces in the mid-hills of Nepal exhibits that losses of nitrogen by leaching ranges from 0 to 63.5 kg N/ha which depends upon location and the form of applied nitrogen (Pilbeam et al., 2004). Difference in N fixing ability in inoculated crops is also inevitable. Partial amount out of total N requirement of wheat could be activities of nitrogen fixing bacteria. They even can increase wheat growth. Excessive use of fertilizer over the decades is found to contribute greenhouse emission ($N_2O$). As nitrogen fixation is gaining importance, it could be an alternative for the use of nitrogenous fertilizer. N fertilizer being the most necessary input in agriculture (Bhattacharjee et al., 2008). Dissolved organic Nitrogen leaching losses from agricultural soils is another problem across the world, as a study done at the upper US Midwest by Hussain et al. (2020) reported 4.5 kg/ha/year which was higher than compared in field of perennial grasses.

Nitrogen fixing endophytes have potential to reduce use of fertilizer inputs by making ammonia available which is used by plants to enhance growth (Mahato & Kafle, 2018). *NifH* gene containing endophytes are found to associate with wheat and fix atmospheric nitrogen (Venieraki et al., 2011). Citrate which are generally considered as toxicants in environment and water are utilized by bacteria as a source of carbon and energy and hereby reduces toxicities of citrate (Kooij, 1977). 16S ribosomal RNA is the component of the 30S subunit of a prokaryotic ribosome. It binds to the Shine-Dalgarno sequence of mRNA. The sequence are used for reconstructing phylogenies. Slower rate of evolution indicates such region are highly conserved over time (Woese & Fox, 1977). V4 region of 16s rRNA sequence differ highly by many nucleotides (Jovel et al., 2016). In this study we aim to isolate, screen and confirm endophytic isolates as bacteria through nucleotide PCR and gel electrophoresis and characterize them in few growth potential terms.

### 1.1 Endophytic bacteria

Microscopic size facilitates endophytic bacteria to harbor inside plant and animal body, though magnitude of availability is yet to be studied (Gardener, 2009). There is enormous progress in symbiotic association between rhizobia and legumes, but pathway to create nodulation of bacteria in leguminous root zones are still unclear (Iniguez et al., 2004). Some show low degree of specificity towards host (Fages, 1994). Some endophytes may enhance host growth, nutrient acquisition and improve plants ability to tolerate abiotic stresses (Jovel et al., 2016). Recruitment of nitrogen fixing bacteria supports to obtain N from atmosphere. Researches shows inoculation is an alternative; being eco-friendly, could replace traditional practice to reduce fertilization costs. Several other studies like study by Wong (1987) is been found to promote growth where the particular bacteria when inoculated on rice seedlings increased the yield by an average of 29.4%. Study performed by Cakmakci et al., (1981) stated that bacterial performance could also be retarded depending upon climate where she has mentioned activity reduction in winter. Such bacteria are also capable to promote growth by suppressing fungus through antagonistic effect (Puri et al., 2018) siderophore production and antibiotics (Dunne et al., 1997). Mechanism of increment in growth bolstered by diazotrophs could be through phytohormone production, stress resistance power build-up, nutrient uptake, improved water status (Creus et al., 2004) and ammonium secretion (Aguilar et al., 1998). Various studies under pot and field conditions suggested endophytes from cereals possess biofertilization property. Inoculation of such bacteria into crops could deliver huge effect in yield and growth (Dalla Santa et al., 2004). The association is helpful in promoting Glick, (1995) Ecological interaction resulting at the root surface can cause deleting effects in the bacterial population (Nehl et al., 1997). Endophytic bacteria can provide systematic resistance to plant disease promoting plant health (Chanway, 2011). Endophytic bacteria offer a solution for improving yield in sustainable manner (Robinson et al., 2016b).

#### 1.1.1 Endophytic microorganisms region of colonization

Microbes colonize at different plant parts like leaves, roots and stems with various density and frequency as suggested by Kuźniar et al. (2020) and higher abundance of microbes was reported in underground parts like roots rather than aboveground plant organs (Robinson et al., 2016b). As roots and leaves of wheat are popular niches for colonization of endophytes where firmicutes were the dominant; relatively abundant proportion in a study Robinson et al. (2016) amongst four bacterial phylums. But bacterial proportion are higher in roots than in leaves as found by Robinson et al. (2016) with similar findings in other crops like cotton, potato, rice and maize (Hallmann et al., 1997). Bacterial community are influenced and get reduced through plant season as mentioned as in (Redford & Fierer, 2008). Thrall et al. (2007) highlighted that long term interaction between plant and endophytes could be a reason for higher abundance of endophytes in low available nutrient field. Study shows that such bacteria thrives well in root, stem and leaves as ectophytes or endophytes (Cakmakci et al., 1981). They are found to be isolated from root interiors, leaves, rhizopsheres (Baldani et al., 1997). Wheat endosperm are great reservoir of beneficial microbes keeping ability to pass generation after generation (Rahman et al., 2018). Robinson et al. (2016b) mentioned wheat microbes can carry huge load of microbes. Growth stage affecting bacterial community research conducted by Robinson et al. (2016a) in wheat concluded bacterial composition is independent with growth stage, soil fertilizer treatment is always variable. Growth condition is related to available nutrients in tissue suggesting endophytes are more likely to adapt in nourished media in laboratory than in soil (Robinson et al., 2016b). Sakurai et al. (2008) mentioned that microbial populations depend, change with root age, amount of light and moisture conditions. Isolation media also influences the composition of bacterial isolates (Germida et al., 1998).

### 1.2 *NifH* in microorganisms

The *nifH* gene encoding enzyme dinitrogenase reductase, genetically conserved in diazotrophic microorganisms (Singh et al. 2013) is a widely used N2 fixing marker for investigators. Nitrogenase enzyme complex is regulated by organized operon forming nif regulon which is one of the most conserved regulon in nature (Gaby & Buckley, 2014). Nitrogen fixation is carried out by metalloprotein enzyme (Aharonovich et al., 2007) nitrogenase that consists of two subunits; dinitrogenase, tetramer of Molybdenom and iron cofactor and dinitrogen reductase (Angel et al., 2018) encoded by contiguous natured nifHDK operon (Choo et al., 2003). Ribbe et al. (1997) discussed nitrogenase linked with monoxide reductase in *Streptomyces thermoautotrophicus*.

### 1.2.1 *NifH* gene and microorganisms

Diverse *nifH* gene containing microbes were isolated from termite guts and methanogens (Ohkuma et al., 1996). The gene is present and widely conserved throughout evolution amongst archaea and eubacteria (Choo et al., 2003). Singh et al. (2013) isolated and characterized by the *nifh* gene of 12 genera of heterocystous cyanobacetria. From ecological point of aspect *nifH* carrying diazotrophs carry importance that serve as primary source of nitrogen through fixing nitrogen into available form Fowler et al. (2013), however Zani et al. (2000) mentioned all *nif* genes are not expressed in the environment.

### 1.2.2 *NifH* gene structure and homologous

nifHDK regulon is made up of genes *nifD* and *nifK* that codes alpha and beta subunit of alpha beta subunit of enzyme dinitrogenase (Choo et al., 2003). Chien and Zinder (1994) divides *nifH* homologos in five main clusters whereas a high degree of sequence homology is found with *vnfH* and *anfH* genes (Zehr et al., 2003). Components of enzyme complex; heterotetrameric core or dinitrogenase subunits are encoded by *nifD* gene and *nifK* gene Gaby and Buckley (2014) binds atmospheric N2 and transfers electrons, whereas dinitrogenase reductase contains iron cofactor composed of identical subunits encoded by *nifH* gene (Aharonovich et al., 2007).

### 1.2.3 *NifH* primers

*NifH* amplification is used in identifying n2 fixing species or organisms that encode nitrogenase (Dyble et al., 2002). Angel et al., (2018) mentioned choice of primer for *nifH* can have significant impact in assessing *nifH* gene diversity extent. Besides forward primers like Nh21F, Ueda19F, and F2 and reverse primers like *nifH1*, *nifH3*, R6 and DVV. Primers polF and polR are also best primer pair (Puri et al., 2018) which is found to have successful amplification of more than 19 *nifH* strains (Poly et al., 2001a).

### 1.2.4 *NifH* gene in database

*NifH* is the most sequenced functional gene ranging from 252 base pair to 500; approximately 480 base pair are heavily sequenced followed by 405 base pair (Gaby & Buckley, 2014). The horizontal transfer of *nifH* is believed to come across from Paenibacillus strains though limited genetic data (Choo et al., 2003) differently compared to *nifDK*. Gaby and Buckley (2014) mentioned more than 32,954 *nifH* sequences are aligned and stored in the database with paralogs of few *nifH* genes highlighting such paralogous genes do not always participate in nitrogen fixation. Santos et al. (2012) mentioned approximately 10% of microbes been sequenced have nif gene. *nifH* gene sequence of range 100-500 are available in database (Angel et al., 2018).

## 1.3 Nitrogen fixation

Factors affecting N availability in environment besides N2 fixation are availability of Fe, P and Mo (Wu et al., 2000). Nitrogen fixation and denitrification determines the biological available nitrogen in atmosphere where microbes do fundamental works in making nitrogen available (Zehr et al., 2003). *Pseudomonas stutzeri*, well adapted in flooded soil, found to harbor nitrogen fixing genes in putative genome (Lin et al., 2000) and colonizes well after inoculating in to wheat root (Rediers et al., 2009). Cakmakci et al. (1981) isolated *Klebsiella oxytoca* from wheat roots studying acetylene reduction technique. Mehnaz et al. (2007) *Pseudomonas* isolates isolated from wheat, rice and sorghum is been found as most N fixing bacteria. *A brasilense* isolates is found to be well established with wheat (Bashan & Holguin, 1997) and well adapted to wheat rhizospere or root interior supporting yield (Rothballer et al., 2003). Rilling (2018) reported *Bacillus* and *Arthrobacter* genera dominantly in wheat.

## 1.4 Nitrogen fixation in cereals

The process of symbiotic in Rhizobia is an interaction between two major class of genes *nifH* and *fix* genes (Shamseldin, 2013). Root system of graminae is also an appropriate biological niche for N fixing bacteria, suggested by (Venieraki et al., 2011). Kucey (2011) observed small amounts of N fixation in plants. Elo et al. (2000) found unknown species of nitrogen-fixing *Paenibacillus* spp. Unlike legume-*Rhizobium,* nitrogen fixing endophytes are not confined only in legumes, they are found to colonize densey in roots of cereals. There is a record of inoculation of sugarcane with *Gluconacetobacter diazotrophicus* by (Sevilla et al., 2001). Kalla grass was also inoculated with *Azoarcus* sp. by (Hurek et al., 2002). Freely living bacteria can associate with cereals and even wheat to help fix nitrogen (Venieraki et al., 2011). Compared to non-symbiotic bacteria, symbiotic bacteria like *Rhizobium* association system contribute much higher contribution (Herridge et al., 2008). Similarly, bacillus were dominantly present in sugarcane (Madhaiyan et al., 2011). Gauthier et al. (2000) also supports that *P. stutzeri* is able to fix atmospheric nitrogen. On a study by Soares et al. (2006) in oat *Azospirillum* spp are most abundant endophytic isolates. A study in Canada by Mehnaz et al. (2007) isolated *Azospirillum* spp form maize. Besides, *Bacillus* were also reported by Puri et al. (2018) as dominant endophytes in sweet potato. Aho and RJ (1974) isolated *Klebsiella* from woods. Neilson and Sparell (1976) isolated from pulp effluents (Werner et al., 1974) from marine sample and legumes by (Evans et al., 1972). The comparative study of impact of *Azotobacter* and *Trichoderma* with other fertilizers on maize growth

done in IAAS, Lamjung revealed that association of *Azotobacter* and Trichoderma was observed in biomass with an increase of 149.8% over control while *Azotobacter* had a biomass increase of 68.2% and *Trichoderma* had an increase of 5.9% to that of control (Mahato & Neupane, 2017). Nitrogen fixing endophytes have potential to reduce use of fertilizer inputs by making ammonia available which is used by plants to enhance growth (Mahato & Neupane, 2017b). (Ding et al. (2005) reported nitrogen fixing ability of *Bacillus* spp. isolated from rhizosphere of cereals growing region.

### 1.5 Cel3 gene in microorganisms

The *Cel3* gene or endoglucanase gene was first identified in *Fibrobacter succinogenes* S85 (Crosby et al., 1984) and is conserved among several isolates of *Fibrobacter succinogenes* bacteria. Lin et al. (2000) supported endoglucanase gene cel3 of *Fibrobacter succinogenes* has orthologous nature as inferred from sequence divergent data and 16S rRNA analysis. Cellulase are identified as highly pH and temperature stable enzymes (Ibrahim & El-diwany., 2007). Cellulase are believed to be produced by microorganisms mostly like fungi and bacteria (Sangkharak et al., 2011) having much importance in the textile industry, biomass fermentation into biofuels (Paulo, 1998) and fiber modification are found to be inducible in course of their growth (Sangkharak et al., 2011).

Model fibrolytic species like *Fibrobacter succinogenes* have been extensively used for study of cellulase degradation (Sangkharak et al., 2011), that contains endoglucanase gene *cel3* (Lin & Stahl, 1995). Endoglucanase 3, endoglucanase C and cellodextrinase from *F. succinogenes* S85 belong to members of same A3 cellulase family on the basis of sequence similarities (Gilkes et al., 1991). The *cel3* gene has also been isolated from strains of basidiomycete, *Irpex lacteus* defining the gene as cellulose inducible (Hamada et al., 1999). Endoglucanase and B-glucosidase are considered as main components of the cellulase system (Coughlan & Ljungdahl, 1988). Yagüe et al. (1996) also isolated and characterized cellulase gene *cel1* and *cel2* gene of *Irpex lacteus* to study regulation of gene sequence where it was found to encode exo-type cellulase, Ex1 and Ex-2. Hamada et al. (1999) concluded cellulase genes of *Irpex lacteus* namely, *cel1*, *cel2* and *cel3* were highly similar in their tripartite structure but Hamada et al. (1999) mentioned that the cel3 fragment didn't hybridize with *cel1* and *cel2* whereas Northern hybridization revealed no difference between transcription of *cel1*, *cel2* and *cel3* genes in cell of *Irpex lacteus*. Such gene can be utilized by microorganisms to break primary structure of plant cell wall (Saha et al., 2006).

#### 1.5.1 Structure of *cel3*

The *cel3* gene is most transcribed and expressed as compared to other hydrolase genes like *celF* (Béra-Maillet et al., 2009). *Cel3* coding region consists of six short introns where intron positions are conserved between *cel3* and other relative cellulase (Hamada et al., 1999). DNA and amino acid sequence of the *cel3* gene are divergent among their species as compared to another glycoside hydrolase enzyme cellulase, encoded by gene *cel3*, gene *celF* (Béra-Maillet et al., 2004). But amino acid sequence of *cel3* if deduced was found to have similar sequence with other exo-type cellulase Ex-1 and Ex-2 suggesting further research requirement.

Amino acid catalytic domain comparison between *cel3* homologous to that of exo-type cellulase belonging to glycosyl hydrolase (Yagüe et al., 1996). and homologous with *P chrysosporium* and *A bisporu*s keep similarity of 74% (Chow et al., 1994). Hamada et al. (1999) manifested 79% similarity of amino acid catalytic domain comparison *cel3* gene with *cel2* gene of *Irpex lacteus*. Truncated *cel3* gene in *E. coli* having only C-terminal region of endoglucanase is able to retain catalytic activity of carboxymethyl cellulose as amino acid sequence are conserved in C-terminal domain of cel3 gene (Jiang et al., 2010). *Cel3* has also been isolated, characterized and sequenced from *Agaricus bisporus* through cloning of *cel3* cDNA (Yagüe et al., 1996) that encodes the cellular gene (Chow et al., 1994).

#### 1.5.2 *Cel3* gene homologous

*Cel3* gene has homologous sequence with gene that codes cellodextrinase A *F. succinogenes* and endoglucanase C of *Clostridium thermocellum* (Schwarz et al., 1988). Like *cel3* gene, some other glycoside hydrolase genes present in genomes of several strains of *F. succinogenes* are *celD*, *celE*, *celF*, *celG* (Béra-Maillet et al., 2004) whereas the *cel3* gene is not present in some strains of F. *succinogenes* like HM2 and NR9 (Lin & Stahl, 1995). Chow et al. (1994) reported cellulase gene *cel1* and *cel3* cab expression can be controlled at transcriptional level by crystalline cellulose and (Ptashne, 1988) suggested that the promoter region of both genes might contain cellulose responsive elements easily recognized by activator. Phylogeny analysis of glycosyl hydrolase enzymes encoded by namely *cel1*, *cel2* and *cel3* have evolved independently and clearly classifiable into two groups as exo-type and endo-type cellulase having cel3 alignment better with exo-type cellulase (Hamada et al., 1999).

#### 1.5.3 Commercial production of cellulase

Cellulose being the most abundant biomass, most abundant carbohydrate, crystalline polymers and primary structure of plant cell wall (Saha et al., 2006). Cellulose, degraded by a process called cellulolysis is catalyzed by cellulase enzymes encoded by gene *cel3* (Immanuel et al., 2006). Microorganisms producing cellulase enzymes can be scaled up being important in possible food supplement and pharmaceutical industry (Shanmugapriya et al., 2012). Cellulase production can be modulated by nature of substrate and media containing less complex carboxymethyl cellulose as best substrate assimilated by Isolated microbes containing the enzyme (Shanmugapriya et al., 2012). Yield of enzyme

production depends on a variety of factors like inoculum size, pH growth time, temperature, carbon and nitrogen source (Robson & Chambliss, 1984).

### 1.6 Inoculation with nitrogen fixing bacteria in wheat

Study by Cakmakci et al. (1981) *Klebsiella* inoculated into plants singly as well as with mixing cultures. Single inoculation did not show any significant effect in growth as dry basis. Even no effect was found in younger plants also, but combination of isolates was more effective. Bhattacharjee et al. (2008) suggests that contribution of atmospheric N nutrition to whole nutrition of wheat plant is negligible. Study by Venieraki et al. (2011) reveals wheat soils are well colonized by *Azospirillum* spp but detected 11 isolates capable of IAA production. *Bacillus polymyxa* isolated from wheat roots in Canada, atmospheric nitrogen contributions were 21, 0 48 and 59% respectively. Study by Subba Rao et al. (1979) *Azospirillum* spp inoculated into soil also found an increased effect in wheat. Symbiotic relationship between nitrogen fixing endophytes from *Rhizobiacea* is unique (Wang et al., 2012). Timmusk et al. (1999) also reported inoculation with plant growth promoting rhizobacteria (PGPR) has potential to promote growth in wheat. Iniguez et al. (2004) studied nitrogen fixation status by *Klebsilla pneumoniae*. Co-inoculation of wheat with endophytes keep potential to increase yield and improve grain quality than uni-inoculation (Roesti et al., 2006) where co-inoculation in some case could result decrease in diversity of some bacterial community in crop is reported by Conn and Franco (2004) in wheat. Coombs and Franco (2003), isolated Actinobacteria from surface sterilized wheat (*Triticum aestivum*) roots. Microbes like Actinobacteria are known to produce antibiotics (Berdy J, 1989) which are able to colonize roots of wheat as well as cereal crops (El-Tarabily et al., 1997; Miller et al., 1989). After inoculation with microorganisms in plants plant root exudation differs in term of composition of tiny molecules (Badri & Vivanco, 2009) like carbohydrates, amino acids, vitamins, minerals and phytohormones that signal root vicinity microbes (Chutipaijit et al., 2008), flavonoids and phenolics (Bhattacharya et al., 2010).

#### 1.6.1 Endophytes enhancing biotic and abiotic resistance

Egamberdieva and Kucharova (2009) focused microorganisms which can tolerate extreme conditions should be used for inoculation in agriculture. Interaction between microorganisms and root of plants is important for plant to adapt in environment (Siddikee et al., 2010). Plants produce compounds like proline, polyamines carbohydrates accumulate in plants to tolerate stress (Barriuso et al., 2008; Kerepesi & Galiba, 2000). Yang et al. (2010) mentioned endophytes that colonize root can increase biotic and abiotic stresses, promote growth by increasing accumulation of secondary metabolites inducing systemic tolerance, resisting stress regarding drought, salt, and nutrient deficiency.

### 1.7 Inoculation with nitrogen fixing bacteria in cereals

*Azospirillium brasilense* sp. 7 was inoculated into roots, originally isolated from roots of maize in Brazil (Bhattacharjee et al., 2008). Rennie and Larson (1981) have also reported that field inoculation with *Bacillus polymyxa* increased the straw yield, N content of a nitrogen fixing isolate by 11 and 15%, and grain yield of an isolate by 13 and 6%.

### 1.8 Glucose fermenting ability of endophytic isolates

Glucose is catalyzed to pyruvate via the EMP pathway. Glucose and xylose are most abundant sugar present in plant biomass as described by (Jiang et al., 2010) supporting butyrate production from glucose. *Tetrasphaera*, being the largest and diverse glucose fermenting organism group (Madigan et al., 2005) utilize glucose aerobically and anaerobically (Nguyen et al. 2011). Isolates having enzyme zymase were able to ferment glucose and produce butyric acid. The butyric acid thus produced is a precursor of indole-3-butyric acid. Indole-3-butyric acid is a rooting hormone which is used by plants and seedlings for rooting purpose. The rooting hormone also promotes plant growth by enabling the plant achieve longer root length and absorb nutrients from deeper portion of soil (Nielsen et al., 2012). Immobilizing cells of glucose fermenting microorganism's butyric acid production can be scaled up through bioreactor. Eco-friendly and cost-effective bioethanol as an alternative for bio-fuel can be produced through manipulation of glucose fermenting microorganisms (Jiang et al., 2010). The butyric acid thus produced is a precursor of indole-3-butyric acid (Alain et al., 2002); has application in chemical industries, food and pharmaceutical (Jiang et al., 2010). However, demand for butyric acid is increasing due to property of bio-based ingredients usable in food and pharmaceuticals. *E. coli* can be manipulated to produce 1,3-propanediol from glycerol (Ma et al., 2009). (Shelp et al., 2017) also mentioned butyric acid derived compound GABA has variety of stresses mitigating effect by providing quick response in variety of stresses in plants; focused butyric acid keep applications in perfume production in chemical industries, adding butter and flavors in food sciences, increasing fragrance like aromatic compounds in plants (Yoshimura et al., 2010). Butyric acid is also a precursor of Gamma Amino Butyric Acid (GABA) reported to be identified in potato tuber (Thompson et al., 1953); is an ubiquitous non-protein amino acid involved in protecting plants from abiotic stress functions in producing primary and secondary metabolites (Ramos-Ruiz et al., 2019); acts as signaling molecule that accumulate in plant tissue during stress (Roberts, 2007).

### 1.9 Citrate utilizing ability of endophytic isolates

Because of the presence of citrate-permease enzyme in isolates, citrate present in media is been utilized as carbon source by isolate. Due to reaction citrate was been converted into ammonium salts and finally to ammonia causing increment of pH due to alkalinity. The ammonia thus produced is another source of nitrogen which is utilized by the plants for its growth. Review also suggest that presence of citrate in drinking water can affect human health (Lantos et al., 1969) and presence in water reservoir may be noxious in industrial purposes as suggested by Schubert (1975). Citrates present in the environment is utilized by citrate utilizing microorganisms as a carbon source and release ammonia to its surrounding making available for plants to uptake as source of nitrogen (Hofwegen et al., 2012).

#### 1.9.1 Indole production by endophytes

Besides nitrogen fixing characters in bacteria, Cakmakci et al. (1981) also isolated indole acetic acid producing bacteria from wheat leaf. Biosynthesis of indole acetic acid from tryptophan occurs via three alternative pathways. Puri et al. (2018) also reported indole produced by *Bacillus* spp.

### 1.10 Role of 16S rRNA and Shine Dalgarno sequence in prokaryotes

The end of 16S rRNA binds with the free mRNA which including the sequence 5' – ACCUCC-3'. Ribosomal binding site in mRNA is known as Shine Dalgarno sequence, enabling initiation of protein synthesis by aligning the ribosome with the start codon. Prokaryotes keep such conserved sequence like 16S rRNA generations after generations in as much intact conditions without altering the sequence so it could be utilized for translation by ribosomal complex. Shine Dalgarno sequence is found around -8 base pair upstream of start codon present in messenger RNA. Deletion of Shine Dalgarno sequence doesn't allow particular bacteria to initiate protein synthesis preventing its translation forever (Marín et al., 2012).

### 1.11 V4 region of 16S rRNA

16S rRNA itself is considered as the most conserved region amongst all genes present through the prokaryotic genome. It is the component of the 30S subunit of a prokaryotic ribosome providing SSU structure. Such ribosomal RNA never gets translated into proteins and amino acid sequence. The whole length of 16S rRNA is approximately 1550 base pair and consists of But the V4 region of 16S rRNA is also considered as partial 16S rRNA which has length of approximately 254 base pair. The V4 region is one of the most hyper variable regions of the conserved sequence of bacterial genome. More conservative regions are used to determine the higher- ranking taxa; however more quickly evolving sequences are used for identification of genus or species (Bukin et al., 2019).

The objectives of present study was to isolate and characterize endophytic isolates of appropriate range dilution isolated from seed and roots of wheat. More specifically this study study characters of endophytic isolates isolated from seed and root of wheat, isolate and screen glucose fermenting endophytic isolates containing nitrogen fixing gene (*nifH*), isolate and screen glucose fermenting endophytic isolates containing *nifH* gene and *cel3* gene

## 2 Materials and methods

### Soil sampling site

Two sites were chosen for soil sampling. Soil from barren field of Pokhara-Lekhnath Metropolitan city of Kaski district and agronomy farm of Agriculture and Forestry University, Chitwan district were sampled.

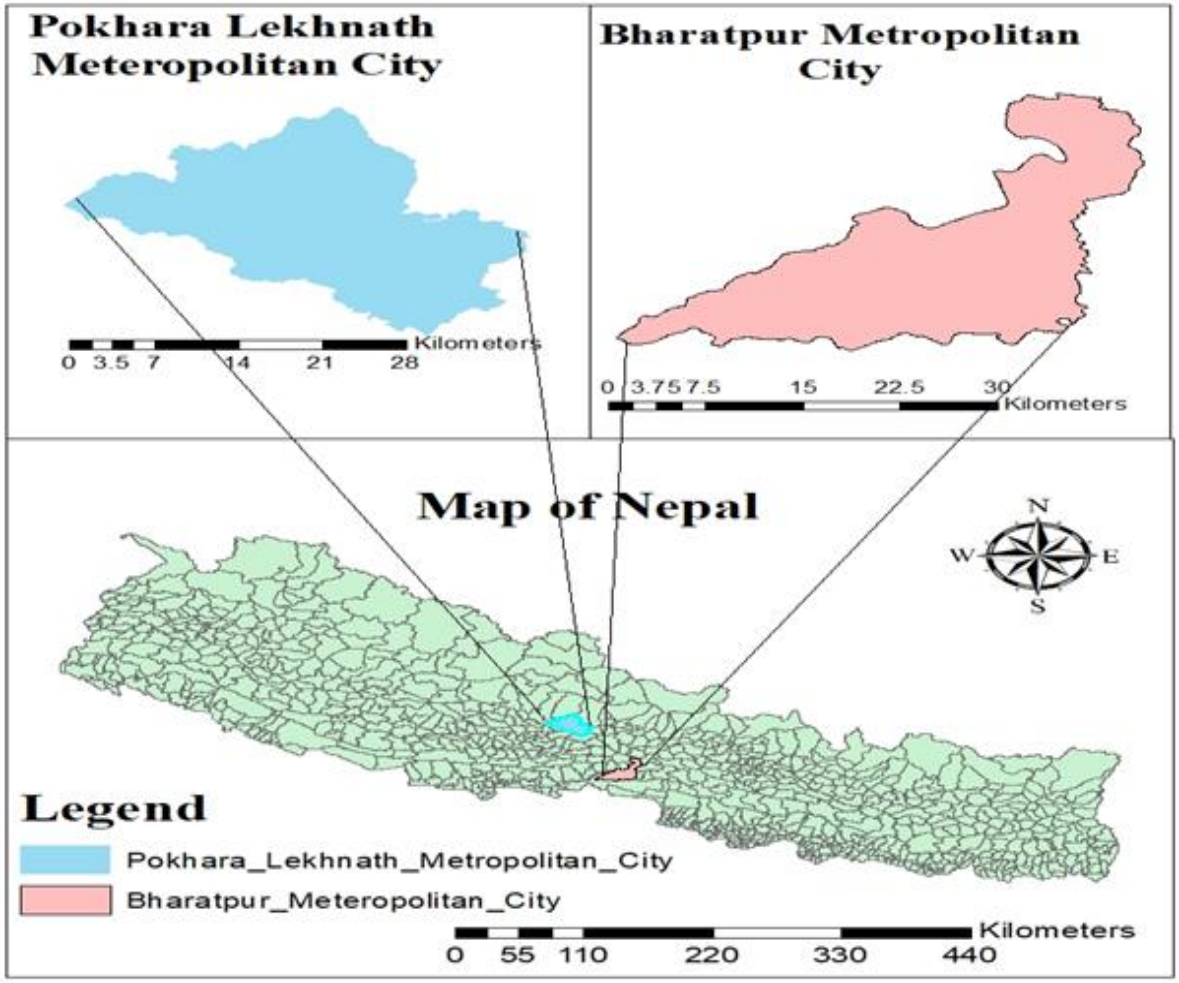



Figure 1: Map of Nepal showing two soil sampling locations

### Soil sampling and soil characters

Soil was sampled from 3 $m^2$ area of the site. From 7 different field sites, 10-inch depth top soil was taken and homogenized properly (Heil & Schmidhalter, 2017). Soil from both field were kept in separate sterilized plastic bags. The bags were labelled and were taken to Center for Biotechnology laboratory, AFU and were shade dried. Eighty gm soil samples were then filled equally in sterilized pots.

### Soil pH reading

Weigh 10 g soil sample is weighted in a beaker and added 25 ml distilled water. Soil and water is mixed with glass rod and waited for half an hour. After 30 minutes reading is taken by pH meter (Orion 410A). Before taking reading pH meter should be calibrated (Thunjai et al., 2001).

**pH- Determination (1:2.5 w/vol)**

Table 1. Soil properties

| SN | Location (District) | Latitude | Longitude | Altitude (masl) | pH |
|---|---|---|---|---|---|
| 1 | Kaski | 28°10'26" N | 84°3'1'' E | 722 | 6.5 |
| 2 | Chitwan | 27°38'43'' N | 84°20'59''E | 180 | 5.6 |

**Processing of samples**

The seeds were surface sterilized and were sown 3 cm depth inside the soil collected from both districts. The seedlings were allowed to grow up to 25 days and total roots were extracted with total pot destruction method without detaching root portion from the seedlings.

Cut opening of pots Whole root mass rescue

Figure 2: Taking sample

**Seed and root surface sterilization**

Similarly, for both root samples, portion with the cotyledon was taken and weighed. The root sample obtained from soil of Kaski and soil of Chitwan soil were 0.94 gm and 0.73 gm respectively. For seeds were washed thoroughly with tap water and rinsed with 99% ethanol for 30 seconds followed by 4% sodium hypochloride (NaOCl) solution for 30 seconds and rinsed finally with sterile distilled water. The samples were hold with sterile tweezers and single drop of final rinsed water was allowed to pour in a solidified nutrient agar medium to confirm surface sterilization and incubated at 37℃ for 24 hours to check microbe's growth.

Figure 3: Seed surface sterilization

The roots were sterilized separately following the above method of complete surface sterilization and crushed with sterile glass rod in separate sterile Eppendorf tubes containing 0.5 ml sterile water. Final sample volume was adjusted to 1 ml by adding distilled water for both root samples.

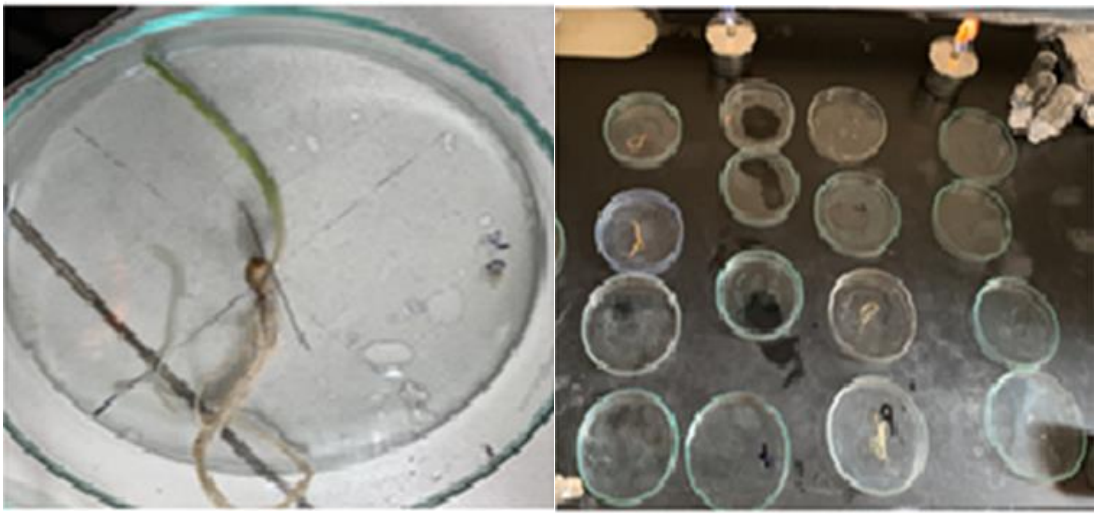


Figure 4. Root surface sterilization

**Preparation of growth media**

With slight modification in (Basu et al., 2015) nutrient agar was prepared as growth media for endophytes. After dissolving the media with sterile distilled water in conical flask, it was autoclaved at 121℃ and 15 psi for 15 minutes. Media was then cooled for few minutes and was poured into sterile petriplates to allow solidifying. After it gets solidification in 4 hrs, the plates were sealed with paraffin tape and incubated at 37℃ overnight to check any contaminations. Contaminated petriplates were discarded and only non-contaminated plates were deployed throughout the whole research works.

Media pouring Media allowed for solidification

Figure 5. Media pouring and setting

**Colony forming unit counts**

Separately, after confirming perfect surface sterilization, it was then crushed in 0.5 ml sterile distilled water keeping in sterilize eppendorf tube and final volume was made to 1 ml adding sterile water. Sample solutions were subjected to serial dilution in sterile Eppendorf tubes. Each eppendorf tubes were labelled. 100 $\mu$l solution was transferred into new tubes containing 900 $\mu$l sterile distilled water for up to $10^{th}$ dilution. From each tube of dilutions, 100 microliter inoculum was taken by micropipette and dropped into surface of each NA media plate. Sterile cotton swab was used to smear smoothly

on each solidified nutrient agar media plates. Each petri-plate was labelled. Swabbed plates were allowed to incubate at 37 ℃ for 48 hours. Growth over the surface of media was recorded for each plates for all dilutions. A single plate having growth at appropriate colony number of 20-250 (Tomasiewicz et al., 1980) were chosen for further study. One plate was chosen each from three samples i.e. root sample P, root sample R and seed sample ds.

Figure 6. Tube with original stock

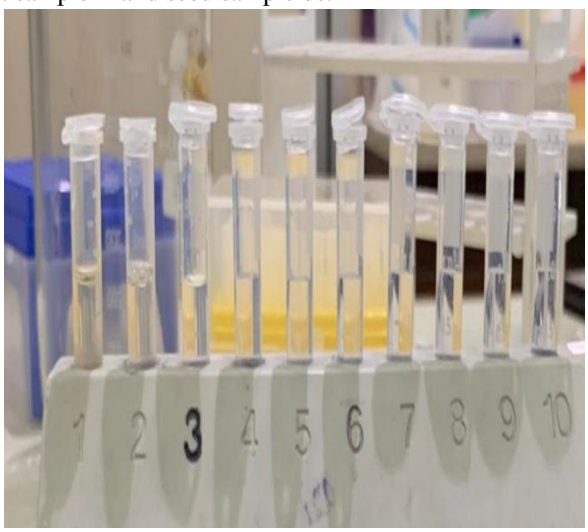



Figure 7. Serial dilution ($10^{-1}$ to $10^{-10}$)

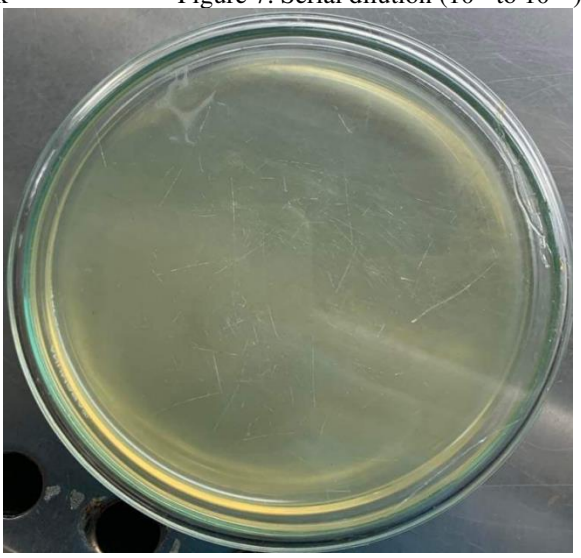


Figure 8. Petri plate with no growth

**Isolation of endophytic bacteria**

Separate nutrient agar plates were assigned for streaking. For first round of isolation of pure colony, we picked the colony and streaked into freshly prepared nutrient agar media. Similarly, we repeated streaking for all isolates. Then we sealed all plates with paraffin tape and incubated overnight at 37℃. To enable to create a perfect colony unit from 1$^{st}$ round, we carried out second round of isolation into new nutrient agar media plates. In this manner, we streaked 47 colonies from seed sample P, 21 colonies from root sample R and 36 colonies from seed sample ds as per mentioned in results. Finally, after sealing with paraffin tape, all petri-plates were incubated overnight at 37℃.

Figure 9. Streaking of colony for isolation

**Preparation of nutrient broth for pure culture**

Nutrient broth was mixed with distilled water and heated until it gets dissolved completely. After cooling, 50 µl media was poured into Eppendorf tubes and sealed. The tubes containing broth were autoclaved at 121℃ and 15 psi for 15 minutes and after cooling for few minutes. The broth was incubated to check any contaminations present inside. Every bacterial isolate resulted after 2nd round streaking was individually inoculated into separate nutrient broth tubes with help of inoculating loop. Plates were kept in shaker incubator (50 RPM) at 37℃ for 2 days.

**Preparation of glycerol stock**

Autoclaved glycerol was diluted with sterile distilled water to make 50% glycerol. 500 microliter of inoculated nutrient broth containing isolates was poured over the tube containing 500 microliter 50% glycerol making v/v ratio of 1:1. Tubes were sealed with paraffin tape. The stock was stored at -80℃ for further process and study.

**Morphologies of isolates**

To study morphology each isolate was streaked with sterile inoculating loop in Z fashion. We carried out morphology study of all one hundred and four isolates. Colonies were categorized on the basis of colony color into four aspects like pale yellow, bright yellow, bright white and pale white.

**Triple sugar fermentation study**

To study triple sugar fermenting behavior, Triple Sugar Iron (TSI) media was used according to procedure explained as (Lehman, 2005). The media was weighed and mixed with sterile distilled water and heated until it gets dissolved. It was then poured into test tubes, which were sealed to air tight with cotton swab and aluminum foil and autoclaved at 121℃ and 15 psi for 15 minutes.

Tubes were taken out, allowed to cool and kept in slanting position to form solidified slants for 4 hours. Tubes were incubated at 37℃ for 22 hours to check any contaminations. After confirming no contaminations, inoculums from corresponding glycerol stock were used directly to inoculate with the help of inoculating loop into the TSI slant medium. To check the color changes and result, tubes were re-incubated at 37℃ for 24 hours.

Table 2. Interpretation of media color

| Color (Slant/Buttom) | Interpretation | Chemical production |
|---|---|---|
| Red /Red | Glucose, lactose and sucrose non fermenter | Alkaline/alkaline |
| Red/yellow | Glucose fermentation only, | Alkaline/acid |

| | | |
|---|---|---|
| Yellow/yellow | Fermentation of all sugars | Acid/acid |
| Splitting/bubbles | Breakage due to gas production | CO2 and H2 |
| Blackening at bottom of slant | Gas (+ or -) | Presence of H2 inside the mediu |

**Citrate utilization study**

For study of citrate utilization by bacteria as per (MacWilliams, 2009), Simon Citrate Agar was mixed with sterile distilled water and heated until dissolution. After cooling for few minutes, the media about 8-10 ml was poured into each one hundred and four separate tubes and sealed with sterile cotton swab and closed with aluminum foil. Each tubes containing media were autoclaved 121℃ and 15 psi for 15 minutes. Tubes were taken out, cooled for 10-15 minutes and kept in slanting position to solidify for 3-4 hours. Isolates from the glycerol stock were inoculated in to prepared SCA medium tubes with help of inoculating loop. Tubes were then sealed with sterile cotton swab and aluminum foil. After labelling with corresponding isolates, tubes were allowed to incubate at 37℃ for 22 hours and waited for resulted color changes.Because of the presence of citrate-permease enzyme in isolates, citrate present in media is been utilized as carbon source by isolate. Due to reaction citrate was been converted into ammonium salts and finally to ammonia causing increment of pH due to alkalinity. The change in pH led to convert green color media into bromothymol blue as shown in results below (MacWilliams, 2009).

**Indole production study**

For study of indole production, motility indole ornithine medium based on procedure of (MacWilliams, 2012) was utilized. Mixing motility indole ornithine media with sterile distilled water heated until it gets dissolved. Motility indole ornithine media was poured on each tube and sealed with sterile cotton swab and aluminum wrapper. After autoclaving motility indole ornithine media, tubes were taken out and it was allowed to cool for 1 hour. Inoculum from glycerol stock was used to inoculate into corresponding tubes with the help of inoculating loop. Tubes were sealed to make air tight and allowed to incubate at 37℃. Media after 44 hours of incubation was mixed with few drops of Kovacs reagent to yield either yellow or pink rings at the top of the tube, where yellow ring indicates negative indole production and pink rings indicates positive reaction. Kovacs reagent reacting with alcohol layer present at the top layer of tube gets converted into pink/red ring color due to presence of color present in the media. The red color ring formed at the top of tube is Rosindol red which is formed by reaction of produced tryptophan been converted into indole, and the reaction is catalyzed by tryptophanase enzyme present in the particular isolates. Isolates not having tryptophanase enzyme wouldn't be able to produce Rosindol red ring at the top of media layer (MacWilliams, 2012).

**Genomic DNA extraction**

Bacterial DNA was extracted with method mentioned as (Pui et al., 2011) with slight modification. One milliliter of Nutrient Broth (M3821, HiMedia, India) was taken in eppendorf tubes. Isolates was inoculated from glycerol stock in to broth inside eppendorf tube followed by incubation at 37℃. Overnight growth broth was cooled at 4℃ for 15 minutes and centrifuged at 13500 rpm for 4 minutes. Supernatant was removed and the pellet was suspended in 500 $\mu$l nuclease free water (AM9932, Ambion). Solution was boiled at 97℃ for 20 minutes and immediately cooled at -20℃ for 15 minutes prior to centrifugation at 13500 rpm for 4 minutes. Finally, supernatant containing genomic DNA was transferred in sterile eppendorf tubes and used for PCR amplification after quantification through gel electrophoresis and spectrophotometer reading.

**Quality checking and quantification of template DNA**

Quantification of DNA extracted was done at 260 nm and 280 nm for determination of sample concentration and purity (X. Li et al., 2014) using nano-drop (Q5000 UV-Vis spectrophotometer). 1$\mu$l of DNA sample was loaded to determine quantity of DNA. With help of Q500 V6.0.2 software the quantity and purity of DNA were calculated. Only DNA samples of 260/280 ratio range from 1.8-1.9 were used for PCR. DNA samples below 1.8 and 2.0 were discarded and DNA was extracted again for such isolates. Highly concentrated DNA was diluted to 120-150 ng/$\mu$l. Concentration of DNA samples for PCR was maintained around 120 ng/$\mu$l by diluting with Nuclease Free Water.

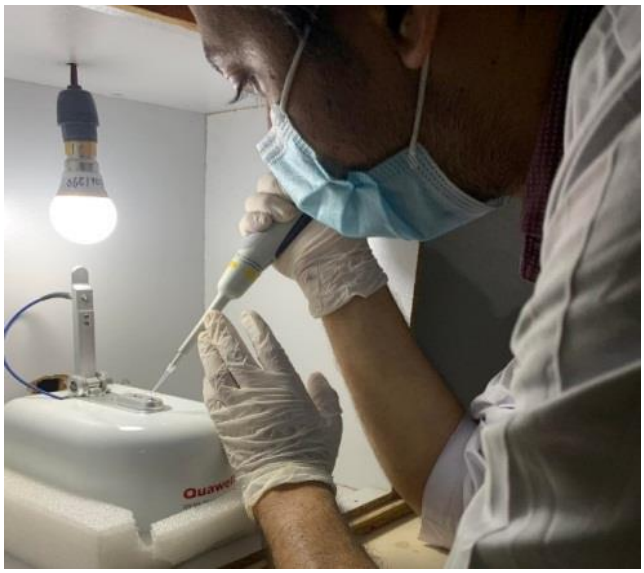


Figure 10. DNA quantification by spectrophotometer

**Primer dilution**

The primer we obtained in tubes was lyophilized for which we diluted each of them to make concentration of about 10 picomoles. For that 1 microliter of concentrated primer is mixed well with 9 microliter of sterile distilled water in sterile tube. The diluted primers are then subjected to subsequent PCR whereas concentrated primers were been stored at -20 ˚C.

**Optimized reaction condition of primers at PCR**

For PCR, following reaction conditions as mentioned as in (Poly et al., 2001b) for *nifH*, (Weisburg et al., 1991) for 16S rRNA, (Béra-Maillet et al., 2004) for *cel3* gene was applied.

Table 3. Optimized PCR conditions

| Genes | Annealing temperature (˚C) | Annealing time | Cycles |
|---|---|---|---|
| *nifH* | 55 | 1 minute | 30 |
| *cel3* | 56 | 45 seconds | 31 |
| 16S rRNA | 56.9 | 30 seconds | 40 |

Figure 11. Loading PCR reagent

***nifH* gene PCR**

Similarly, concentration of DNA samples to subject PCR followed by sequencing was maintained over 100 ng/μl. And concentration of PCR product to be sequenced was maintained over 25 ng/μl. Total volume of PCR run was 15 μl. Primers used is mentioned as in (Poly et al., 2001b).

- For control negative, sterile water in place of DNA
- For control positive of *nifH* gene, DNA extracted from root nodule forming bacteria

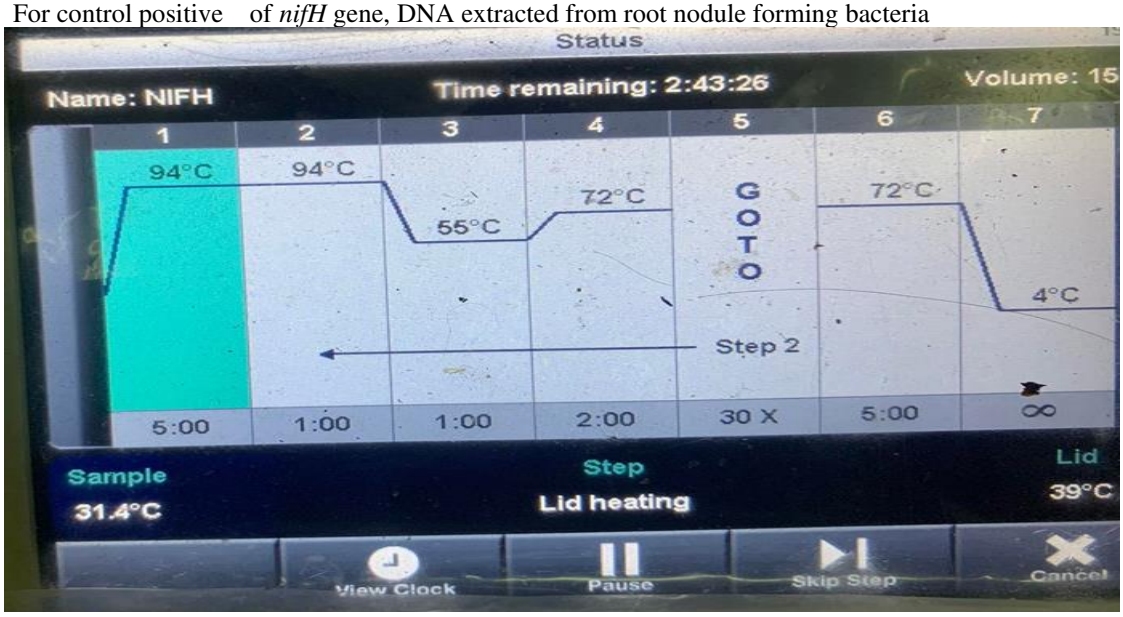



Figure 12. PCR run of *nifH* gene

Table 4. *nifH* gene primers

| Primers name | Type | Primer sequence (5'-3') | Concentration used | Amount used (μl) |
|---|---|---|---|---|
| PolF | Universal | TGCGAYCCSAARGCBGACTC | 10 picomoles/μl | 1.0 |
| PolR | Universal | ATSGCCATCATYTCRCCGGA | 10 picomoles/μl | 1.0 |

***Cel3* gene PCR**

DNA concentration of samples for PCR was maintained over 100 ng/μl. Concentration of PCR master-mix was 2x. Total volume of PCR run was 15 μl. Primers used is mentioned as in (Béra-Maillet et al., 2004).

- For control negative, sterile water in place of DNA
- For control positive of *cel3* gene, DNA extracted from *Salmonella* spp and *E. coli* (Source: Center for Biotechnology, AFU)

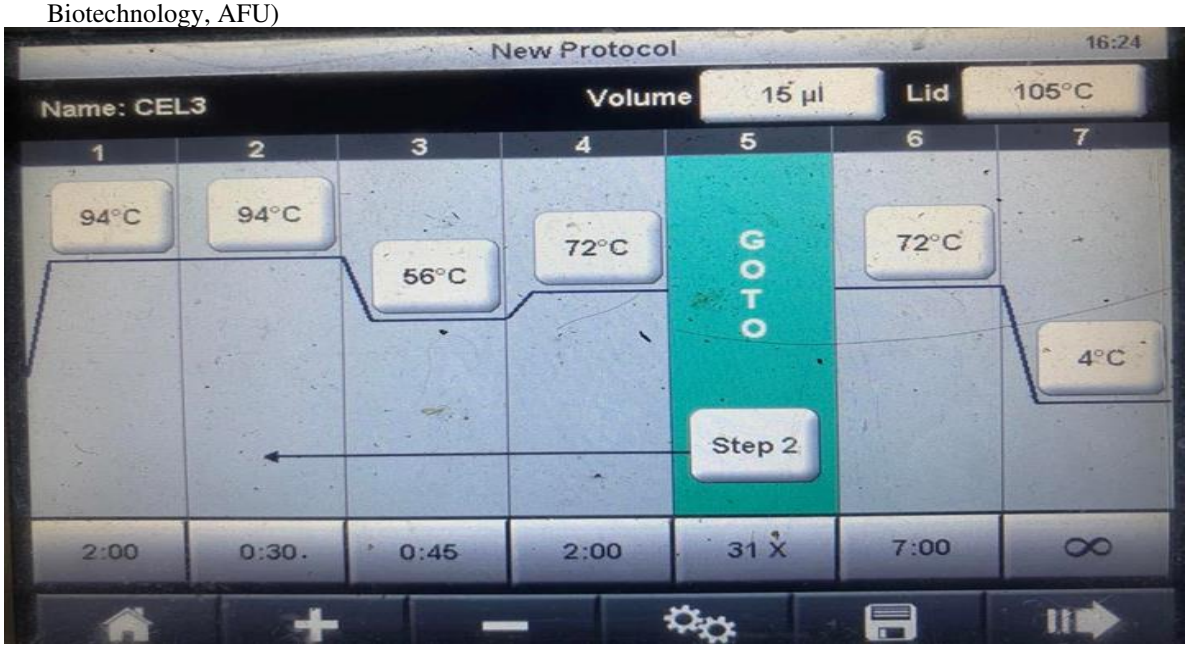


Figure 13. PCR run of *cel3* gene

Table 5. *Cel3* gene primers

| Primers name | Type | Primer sequence (5'-3') | Concentration used | Amount used ($\mu$l) |
|---|---|---|---|---|
| cel3_F | Universal | AGCGATGGTAAGGTCACTGC | 10 picomoles/$\mu$l | 1.0 |
| cel3_R | Universal | AAGGAATCTGGAGGGTATCG | 10 picomoles/$\mu$l | 1.0 |

**Tris Borax EDTA buffer preparation**

| Chemical constituents required | |
|---|---|
| Tris Hydroxymethyl Aminomethane | 109 gm |
| Hydrochloride (FisherBiotech): | depends |
| Boric Acid (Sigma) | 55gm |
| EDTA disodium Salt (Himedia) | 4.65 gm |
| Deionized water | 800 ml |
| Final volume: | 1000 ml |
| Strength | 10x (pH: 8.3) |

Here we would obtain Tris Borax Ethelenediaminetetraacetic acid buffer of strength 10x, so the buffer strength was tilted to 0.5x by using formula N1V1 = N2V2. Constituents were weighed and dissolved inside a 1000 ml flask containing 800 ml sterile distilled water with magnetic stirrer inside the flask. pH of the solution was measured throughout the whole process by adding NaOH to increase pH and HCL gradually to adjust final pH up to 8.3. After achieving pH around 8.3, volume of deionized water was added to make final volume 1000 ml and pH of 8.3 simultaneously (Doyle, 1991).

**1.5% Agarose gel preparation**

Protocol been followed is mentioned in (Voytas, 2000). 3 gm of agarose powder was mixed properly with 150 ml 0.5x Tris Borax Ethelenediaminetetraacetic acid buffer and allowed to boil until bubbles inside gets disappear completely. Then 2$\mu$l Ethidium Bromide (EtBr) was mixed properly. When temperature drops 50℃ -60℃, gel was poured in gel casting tray with comb. The hotness of gel was optimized so as to prevent quick solidification in flask. 1.5% agarose gel was prepared by dissolving agarose powder in 0.5x TBE buffer and mixing 2$\mu$l Ethidium Bromide (Etbr).

**Screening of *nifH* gene containing isolates**

The PCR products were dyed and loaded in agarose gel electrophoresis. After passing 80 voltage electric current for 1.30 hours the gel matrix was subjected to UV ray for gel documentation. Appearance of band at range of approximately 400 bp due to amplification reaction caused by PolF and PolR was assigned as *nifH* gene containing isolates similarly as mentioned in (Poly et al., 2001b).

**16S rRNA PCR**

PCR mixture was prepared by mixing Nuclease free water (Qiagen), Hot Start *Taq* 2x master mix (New England BioLab), forward and reverse primers together with DNA template. PCR reaction as mentioned as in (Weisburg et al., 1991) was carried out with a pre-run at 95℃ for 10 min, 30 s at 95℃, 30 s at 56℃, 60 s at 72℃ for 30 cycles and final run at 72℃ for 10 min. The PCR product was stored at -20℃. Total volume of PCR was 30 $\mu$l. Negative control was made by adding all reagents and distilled water in place of DNA template.

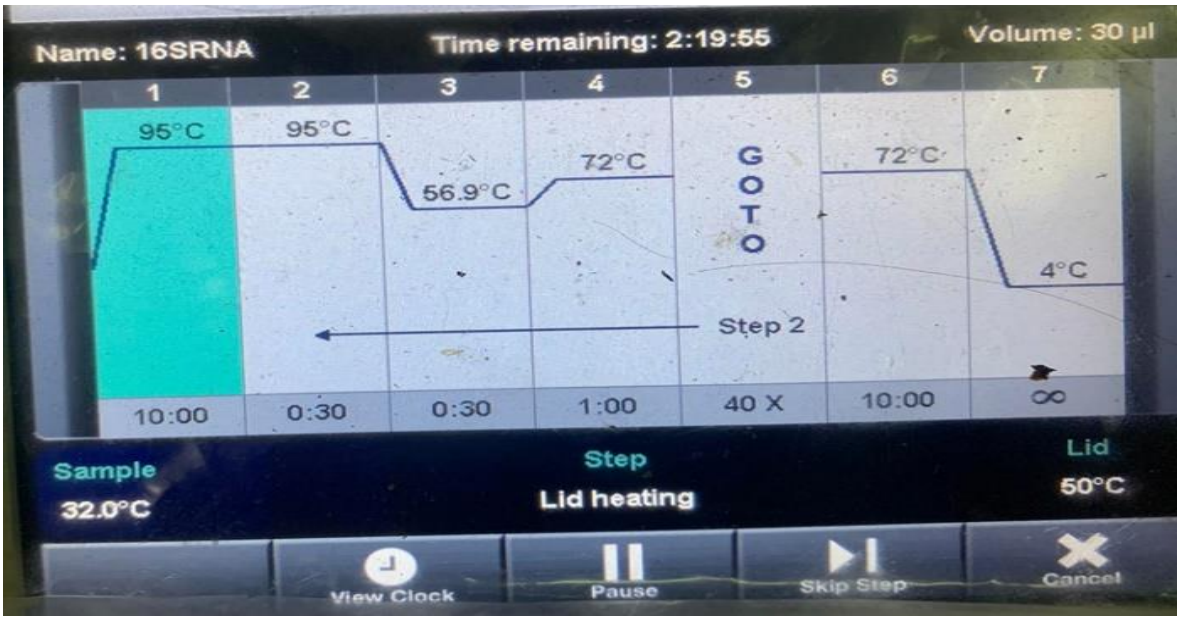


Figure 14. PCR run of 16S rRNA gene

Table 6. 16S rRNA gene primers

| Primers name | Type | Primer sequence (5'-3') | Concentration used | Amount used ($\mu$l) |
|---|---|---|---|---|
| 6F | Universal | GCAACGCGAAGAACCTTACC | 10 picomoles/$\mu$l | 2.0 |
| 1492R | Universal | GGTTACCTTGTTACGACTT | 10 picomoles/$\mu$l | 2.0 |

**Agarose gel electrophoresis**

PCR product was dyed with loading dye and loaded into the wells of 2% TBE agarose gel matrix (0.5x) stained with Etbr (Voytas, 2000). Electric current was passed to allow PCR product separation in 0.5x TBE running buffer. 520-550 bp length gene bands were clearly visualized under UV ray through gel documentation system.

**Statistical analysis**

All the observed data were subjected to excel entry and analysis was done with Microsoft Excel 2016 for table's preparation, graphs insertion and percentage calculation.

**Diversity analysis by Shannon Weiner index**

The Shannon index also known as Shannon entropy, Shannon- Weaver index, Shannon-Weiner index and Shannon's diversity index, has been popularly used in calculation of diversity index (Spellerberg & Fedor, 2003) which is calculated as follows.

$H' = -\sum_{i=1}^{R} pi * \ln * pi$

Where pi is the proportion of the traits observed,

I.e. $pi = \frac{\text{population bearing given trait}}{\text{total population}}$

***Evenness***

The distribution of traits over the population under study can be defined as the population evenness, denoted by "J" (Peet, 1974).

$J = \frac{H'}{\ln(S)}$

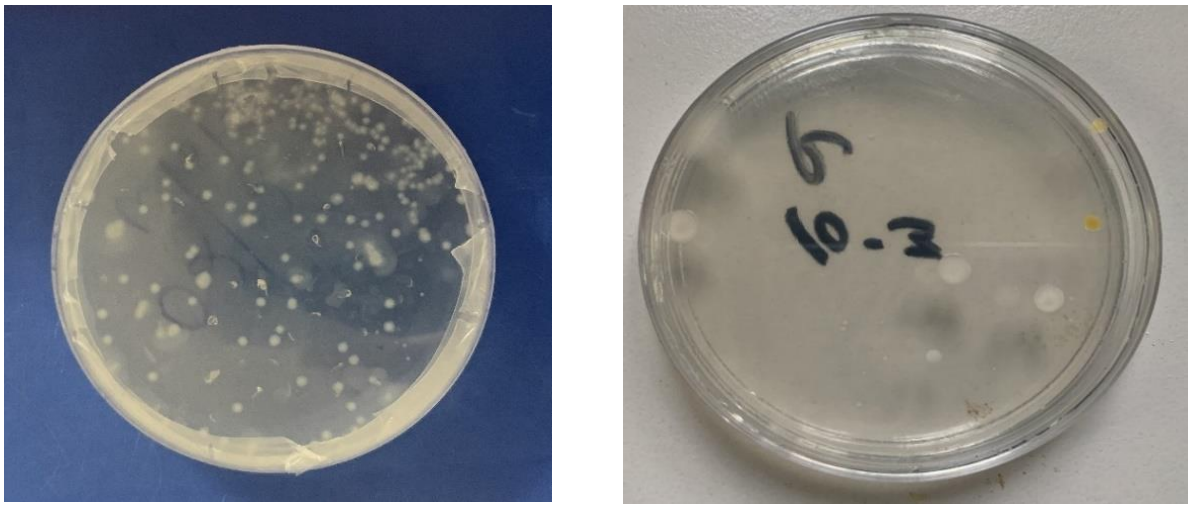
Where, *H'* indicates Shannon Weiner index and S is total number of cases

## 3. RESULTS

### 3.1 Colony forming unit counts

After surface swabbing 100 microliter inoculums in nutrient media, colonies of several colors like pale yellow, bright yellow, bright white and pale white were obtained. Similarly three types of colony shape were obtained like as circular, imperfect circular and wavy. Likewise, several sized colonies were obtained as size range of below 0.1 mm, 0.1 to 1 mm,

and 1 mm to 3 mm and larger than 3 mm. In the plate having $10^{-7}$ dilution from soil root sample P, forty seven colonies were obtained. Similarly, from $10^{-3}$ dilution of root sample R, twenty one isolates were obtained. At same manner, from $10^{-8}$ dilution from seed sample ds, thirty six colonies were obtained. Finally, we got to make our samples to one hundred and four. Pale white colonies of dominant color with more than 20% of total isolates and few colonies of bright white color were obtained where imperfect circular colonies as dominant shape followed by circular shaped colonies with more than 22% and 10% respectively were obtained. And, similarly colony size of 1 mm to 3 mm were found to be dominant compared to other sizes of colonies.




CFU from root sample R in $10^{-1}$ dilution  CFU from root sample R in $10^{-3}$ dilution

Figure15. FU from root sample R

**3.2 Biochemical study on total isolates**

**Citrate utilization study**

The change in pH led to convert green color media into bromothymol blue as shown in results below. 9.5% of total isolates were citrate utilizers but none of those citrate utilizers were *nifH* gene containing isolates. All citrate utilizing isolates under our study belonged to root sample P and isolates namely P11, P13, P14, P16, P20, P21, P24, P34, P35, P41 were found to have positive citrate utilization response, where none of isolates from root sample R and seed sample ds had citrate utilizing isolates.

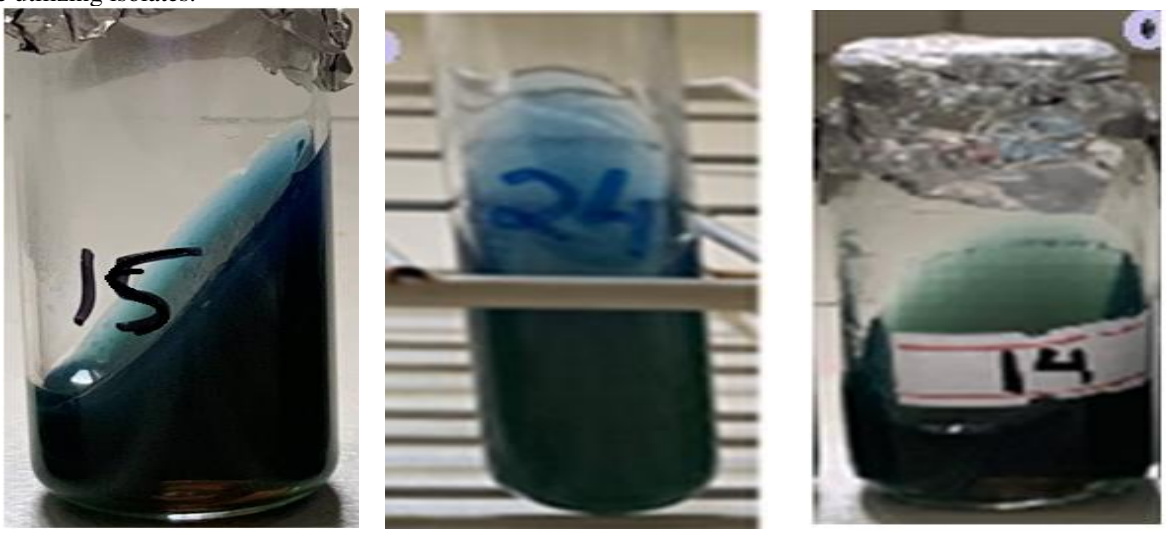



Figure 16. Control positive, citrate utilization positive and citrate negative

**Indole production study**

The red color ring formed at the top of tube is Rosindol red which is formed by reaction of tryptophan been converted into indole catalyzed by tryptophanase enzyme. In our study, all isolates from glucose fermenting group and glucose non

fermenting group manifested negative indole production activity suggesting that none of isolates were having tryptophanase enzyme encoding genes.

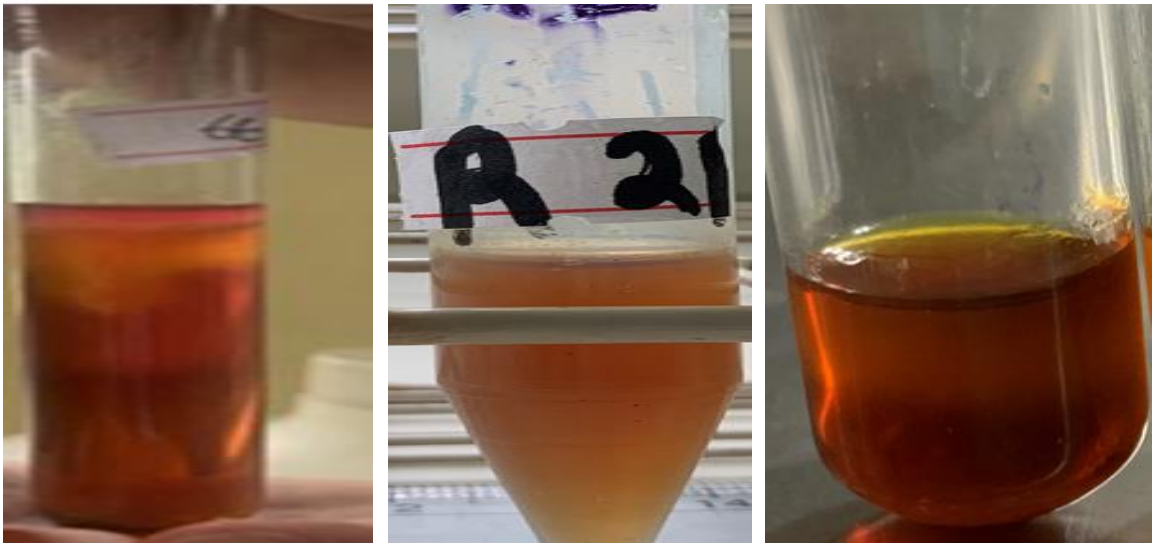



Figure 17. Control positive indole production negative and control negative

**TSI fermentation study**

Forty nine isolates (46.6%) of total isolates were found to ferment glucose where 33.3% of *nifH* containing isolates were glucose fermenters. Similarly, isolates having enzymes invertase and Beta-galactosidase enzyme were able to ferment sucrose and lactose respectively. Two isolates amongst one hundred and four isolates namely P26 and P44 were able to ferment sucrose and lactose where those 2 isolates weren't containing *nifH* gene.

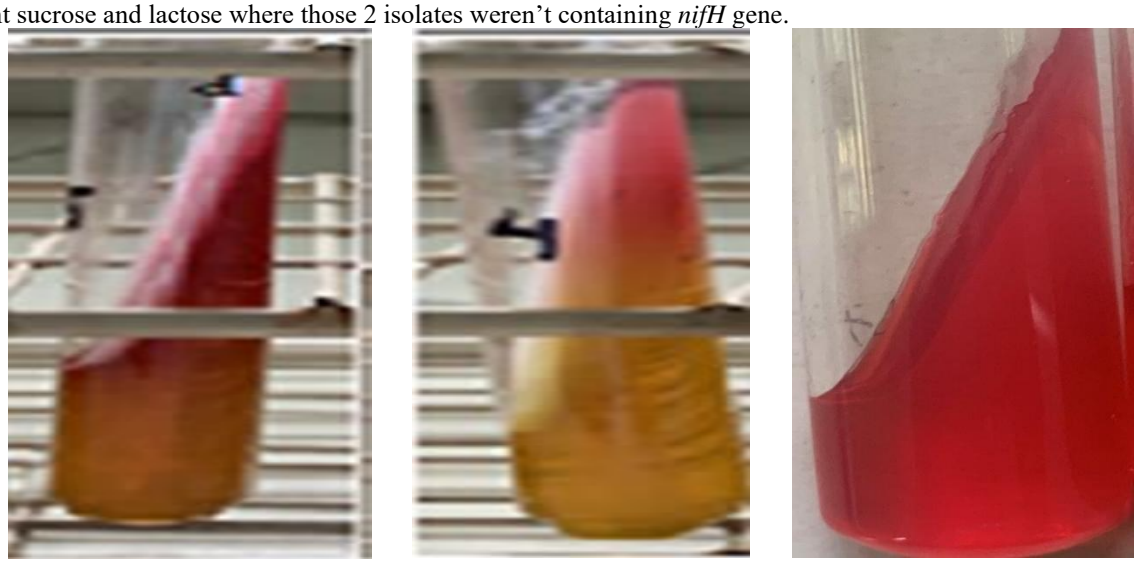


Figure 18. Control positive, glucose fermentation positive and control negative

Similarly, nineteen isolates out of one hundred and four isolates i.e. 19.04% of total isolates were hydrogen sulfide gas producers. All hydrogen sulfide producers were been isolated from root sample R where none of isolates been isolated from root sample P and seed sample ds were hydrogen sulfide gas producers. Few isolates namely R1 and R7 weren't able to produce hydrogen sulfide gas inside the medium.

Figure 19. Control positive, H2S production positive and control negative

**Isolates frequency on the basis of biochemical tests**

In sum, the graph shows ten isolates i.e. 9.5 % of total isolates were citrate utilizers but none of those utilizers were found to contain *nifH* gene. In our study, six and four isolates from glucose fermenting group and glucose non fermenting group respectively responded positive citrate utilization. And there weren't singe indole producing isolates. Forty nine isolates out of total were glucose fermenters whereas only two isolates were fermenting sucrose and lactose. Nineteen isolates i.e. 18.26% of total isolates were obtained to produce hydrogen sulfide.

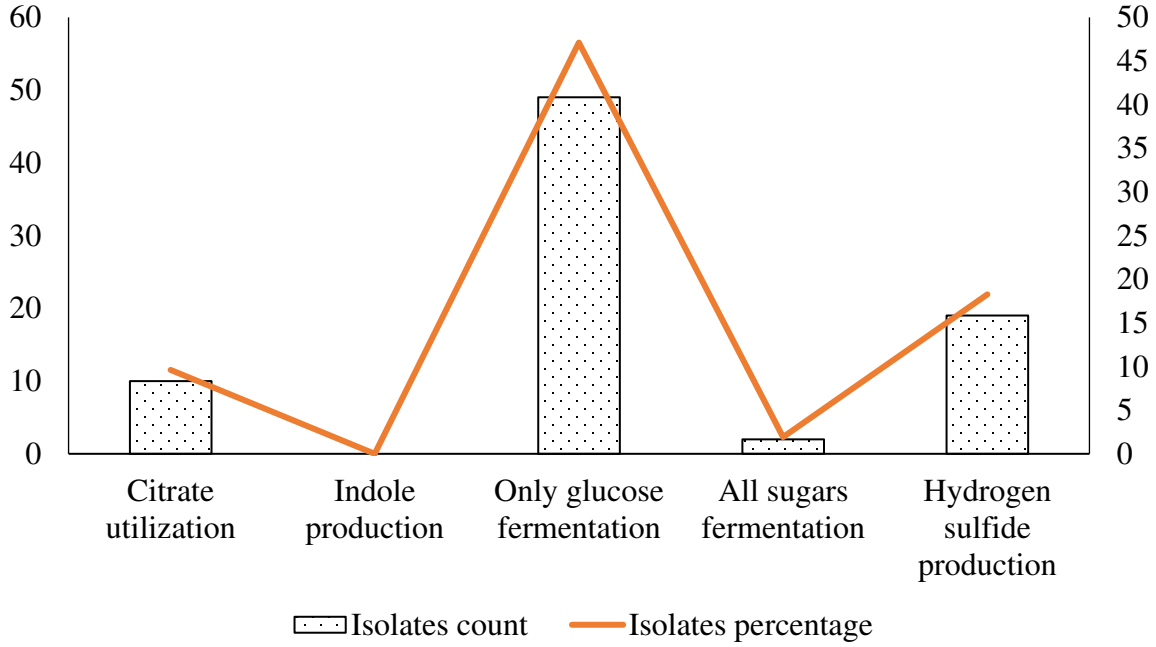
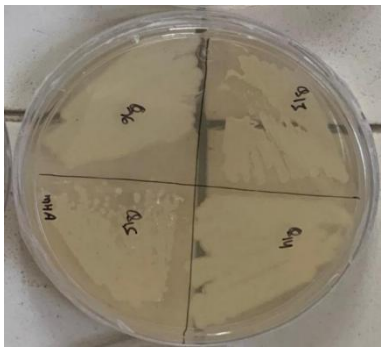


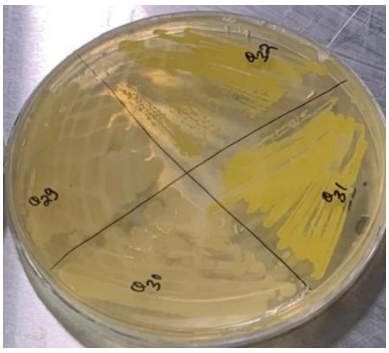
Figure 20. Graphical representation of isolates count and percentage

**3.3 Colony color study**

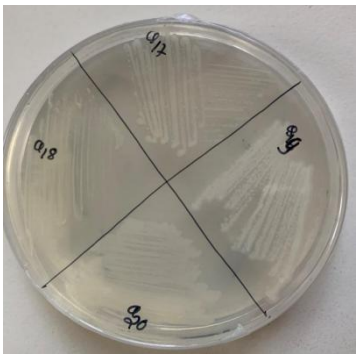
Streaked colonies were grouped on the basis of color too where the total colonies were categorized into pale yellow, bright yellow, bright white and pale white. Pale white color was found to be dominant with more than 20% compared to other colony color. Other colors like bright yellow and bright yellow were obtained at near about equal frequency.



Streaking colony for pale white color study



Streaking colony for pale yellow and bright yellow color study



Streaking colony for bright white colc study

Figure 21. Streaking colony

The pie-chart shows dominant in pale white colony color with around 59% followed by color pale yellow and bright white at near about equal frequency of above 15%.

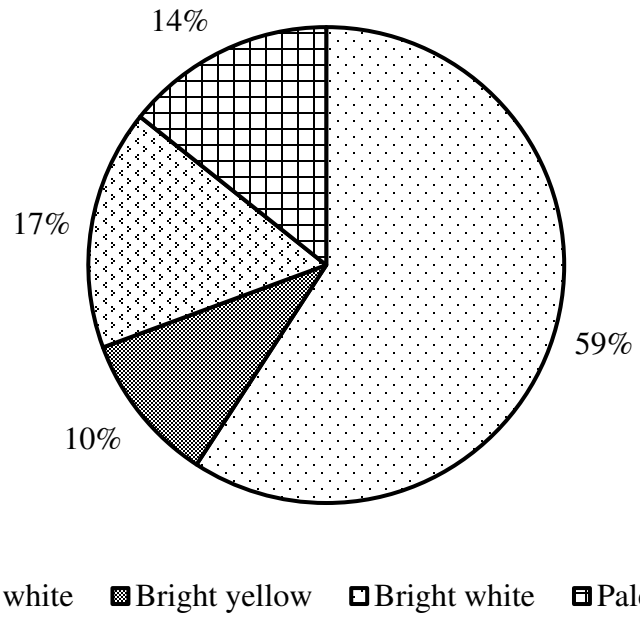



Figure 22. Pie-chart showing percentage of isolates with colony color

**4.4 Colony shape study**

The pie-chart shows there were dominance of imperfect circular shaped colonies with more than 50% followed by irregular shape and circular shaped colonies at near about equal frequency and percentage.

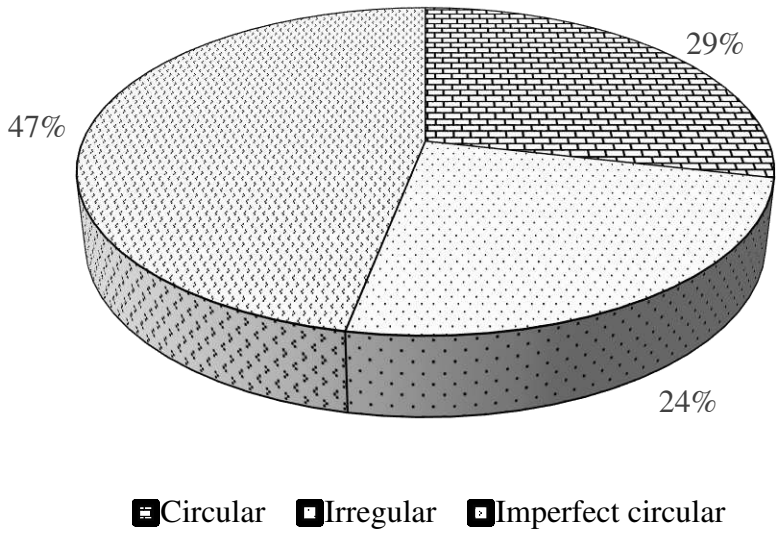



Figure 23. Pie chart showing endophytic frequency with colony shape

**3.5 Colony size study**

The bar graph shows dominant colony sizes of less than 0.1 mm with more than 40% and followed by colony size of 2.0 to 3.0 with about 30%. We obtained colony sized 0.1 to 1.0 mm approximately 20%.

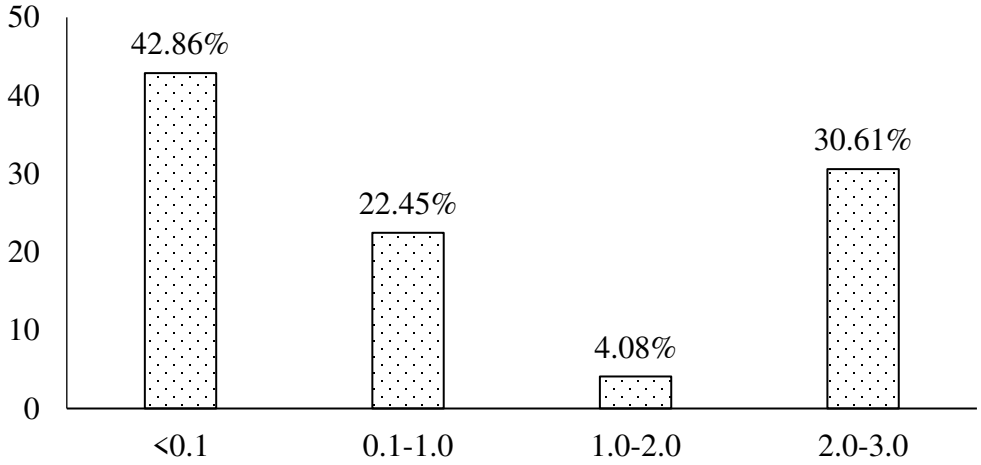



Figure 24. Bar graph showing colony sizes of various range

**3.5.1 Diversity indexes of isolates under study**

The maximum number of diversity in character of glucose fermentation of isolates (0.4754) followed by hydrogen sulfide production (0.4754), citrate utilization (0.3165). Sucrose fermentation and lactose fermentation has least diversity index (0.095), which means that 104 isolates were least diverse in case of sucrose and lactose fermentation.

Evenness value provides information regarding the equality in the distribution of the characters present in the population. Evenness value which equals to 1 means a completely even population i.e. the traits and population is equally distributed. And evenness value near about one means near about complete evenness.

Table 7. Shannon Weiner index and Evenness of the characters of isolates under study

| | Shannon Weiner Index (H') | Evenness (J) |
|---|---|---|
| Glucose fermentation | 0.6914 | 0.9975 |
| Sucrose fermentation | 0.095 | 0.137 |

| | | |
|---|---|---|
| lactose fermentation | 0.095 | 0.137 |
| citrate utilization | 0.3165 | 0.4566 |
| H2S production | 0.4754 | 0.6859 |

**3.6 Quantification of DNA**

The image shows quantification through agarose gel electrophoresis before subjecting DNA for PCR as it shows DNA of appropriate quality and quantity is present for all samples run in agarose gel.

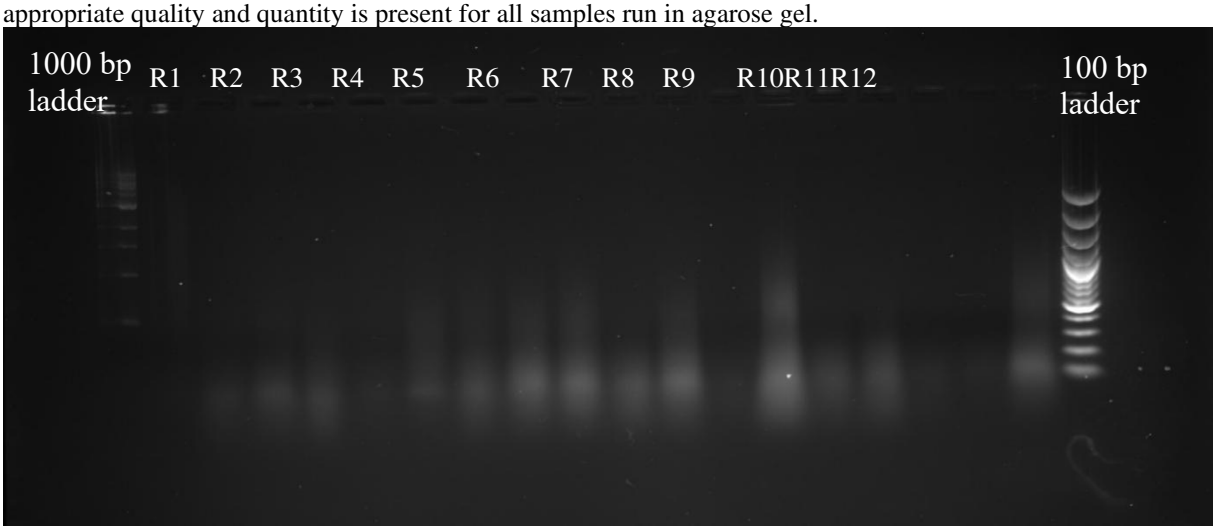



Figure 25. Gel image representing DNA of isolates of root sample R

**3.7 Molecular screening of isolates containing *nifH* gene**

**Molecular screening of control positive containing *nifH* gene**

Three isolates namely ds6, ds7 and ds22 were checked with control positives at the band range of approximately 405 bp. The lane adjacent of control positive belonged to control negative having non PCR amplification due to absent of DNA samples in sterile water.


Figure 26. DNA bands representing *nifH* gene in control positive and three glucose fermenting isolates

**Molecular screening of glucose fermenting *nifH* gene containing isolates**

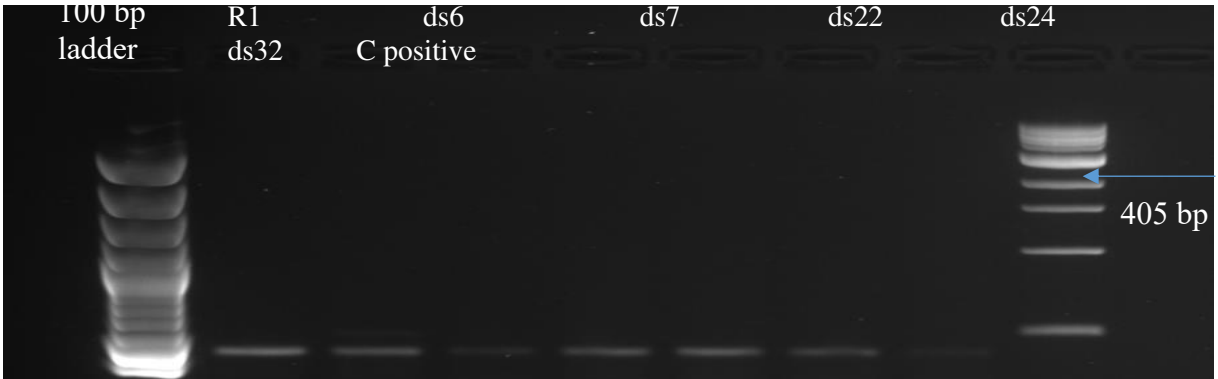


Figure 27. DNA bands representing *nifH* gene presence in glucose fermenting isolates
6 isolates namely R1, ds6, ds7, ds22, ds22 and ds32 as shown as in the figure from glucose fermenting group were detected to contain *nifH* gene, showing band size equal to approximately 405 bp compared to DNA band of control positive.

**Molecular screening of glucose non fermenting isolates containing *nifH* gene**
12 isolates (21.42 %) of glucose non fermenting group manifested positive screening of *nifH* gene range of 405 bp compared to control positive isolate. R7, R8, R9, R14, R17 and R18 were not having *nifH* gene though they belong to glucose non fermenting group.

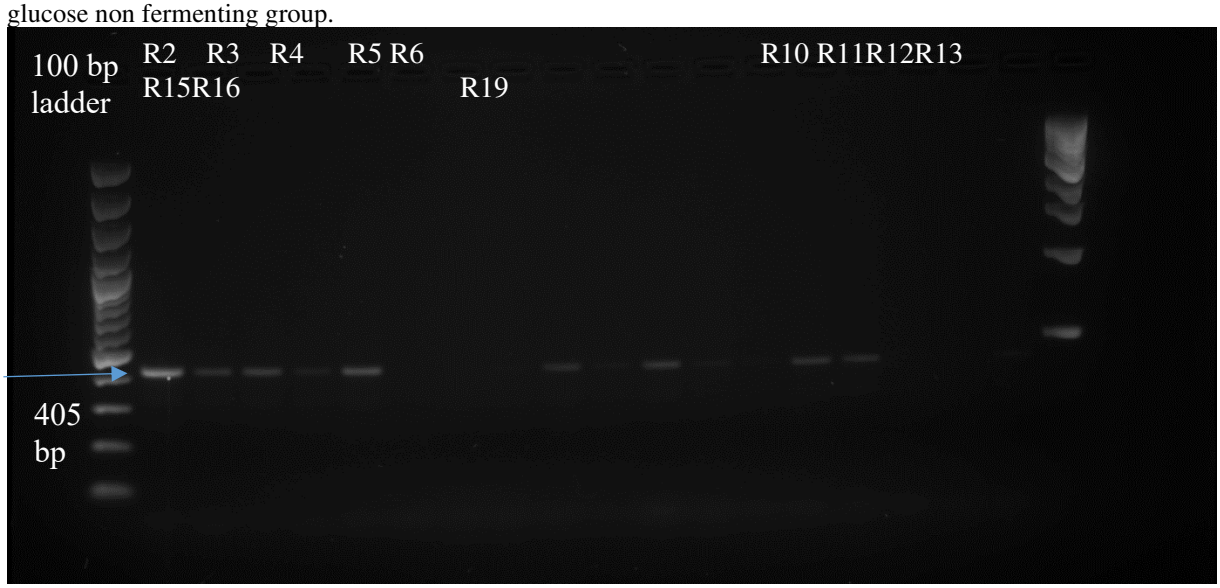



Figure 28. DNA bands representing *nifH* gene in glucose non fermenting isolates

**3.8 Molecular screening of isolates containing *nifH* gene *cel3* gene**
**Molecular screening of *cel3* gene in *Salmonella* spp. *and E. coli***
In the gel image mentioned above, *Salmonella* serovars S7, S83, S89 and *E. coli* serovars E30, E41 and E52 were having *cel3* gene of size 400 and 200 bp as our targeted gene compared with the . But the serovar S60 having *cel3* gene of size approximately below 400 is not our targeted gene size manifesting cel3 gene variant could be of variable size depending upon serovar. Similarly, serovar E71 is not having *cel3* gene of any targeted gene size manifesting that some *E. coli* serovar do not possess any of *cel3* gene on them.

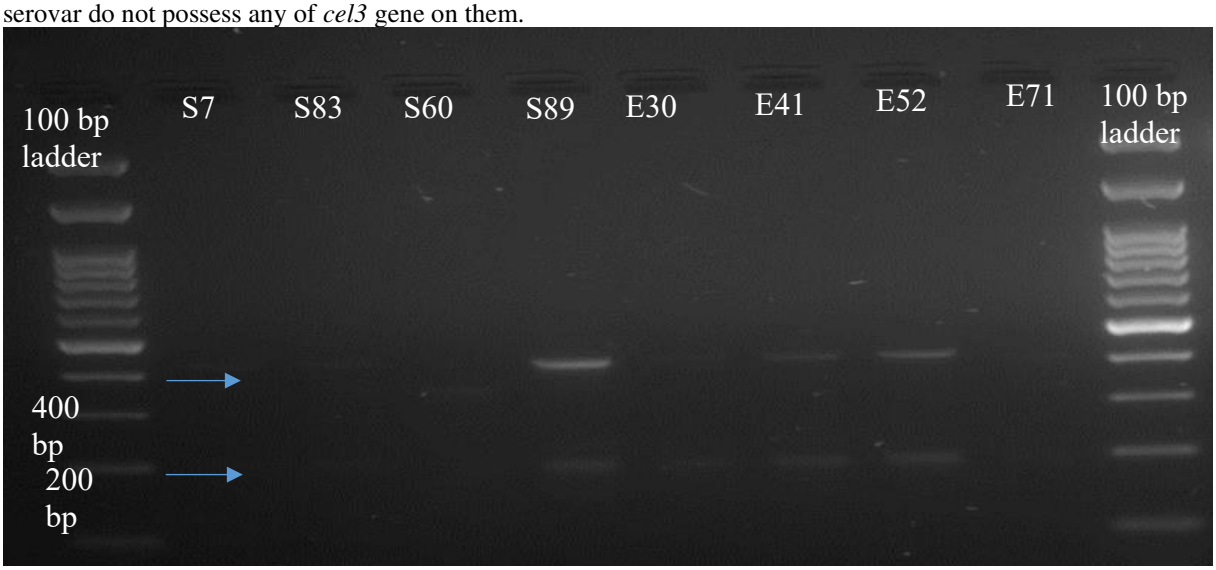



Figure 29. DNA bands representing targeted *cel3* gene in *Salmonella* spp. and *E. coli*

**Molecular screening of *cel3* gene in *nifH* gene containing isolates**

Two isolates namely ds6 and ds7 from glucose fermenting group were containing *cel3* gene of approximately 400 bp and 200 bp whereas 11 isolates were found to have *cel3* gene of approximately 200 bp. Four isolates namely R2, R3, R4 and R6 had targeted *cel3* gene of approximately 400 bp but they belong under non glucose fermenting group.

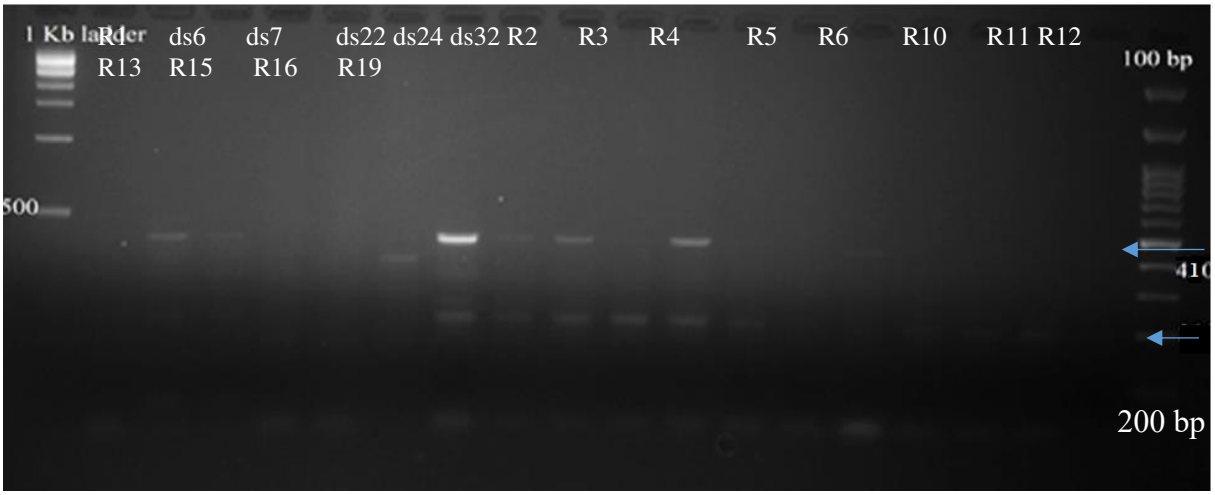



Figure 30. Bands representing *cel3* gene presence on isolates of root sample R

**3.9 Frequency of isolates screened with genes under study**

Compared between root sample P and root sample R, 13 isolates from root sample were obtained to have nitrogen fixing gene whereas five isolates isolated from seed were obtained. Similarly regarding *cel3* gene, four isolates from root sample R had both targeted genes of sizes 400 base pair and 200 base pair, but only two isolates from seed sample ds had both targeted *cel3* gene. None of isolates from root sample P were found to contain neither *nifH* gene nor *cel3* gene.

Table 8. Frequency of isolates screened with genes

| Sample | Molecular screening | |
|---|---|---|
| | *nifH* gene | *Cel3* gene (of both targeted size) |
| Root sample R | 13 | 4 |
| Root sample P | 0 | 0 |
| Seed sample ds | 5 | 2 |

**3.10 Molecular screening of 16S rRNA of *nifH* containing isolates**

As 16S rRNA of all *nifH* gene containing isolates were been successfully amplified and visualized under UV ray through agarose gel documentation. The 18 isolates having *nifH* gene were previously appeared to have colonies as yeast also forms colonies in growing nutrient media. So by utilizing 16S rRNA primers for amplification of the partial gene confirmed presence of 16S rRNA gene on those *nifH* gene containing isolates and confirmed to be bacteria as the gene 16S rRNA is present only in prokaryotes; not present in eukaryotic colony forming organism like yeast ( Li et al., 2011).

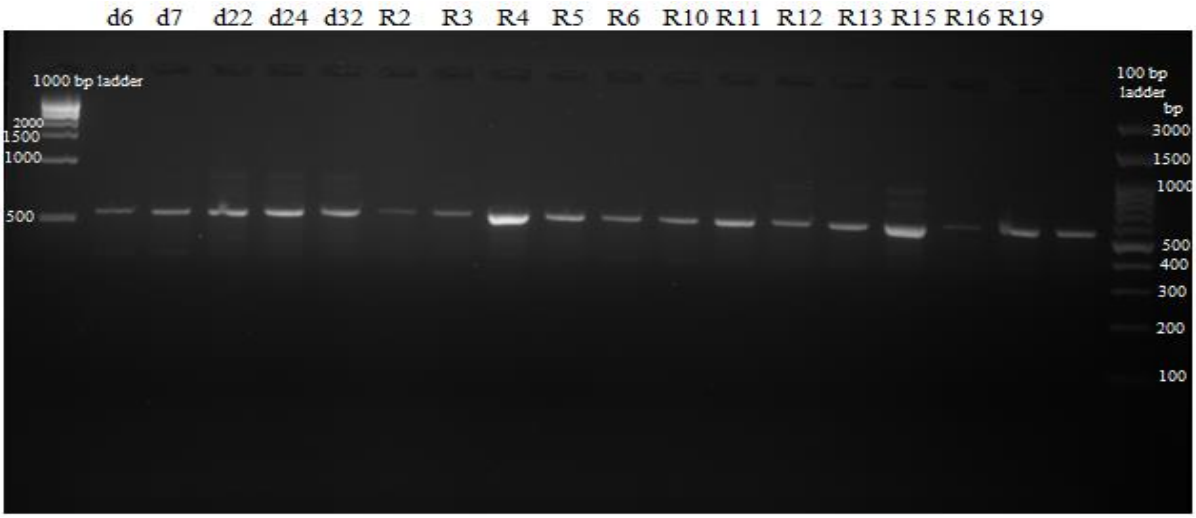


Figure 31. Bands representing 16S rRNA amplification of isolates containing *nifH* gene

## 4. Discussion

The present study was carried out to isolate and screen endophytic bacteria from wheat to study plant growth promoting potentiality in Chitwan and Kaski district of Nepal. To describe naturally occurring microbial populations extracted from different habitats, plate count method could be utilized (Hattori, 1982). Estimate of optimal count can be done and can be narrowed down to search for the best dilution plate saving time at same point (Ben-David & Davidson, 2014). Generally, one can assume that colony forming units are supposed to be distributed equally throughout the solution. Our study is in close agreement with reports on endophytes according to work of Jha and Kumar (2009) who suggested large number of endophytes could be isolated from wheat interior parts. Here we obtained one hundred and four isolates in total.

### 4.1 Factors affecting isolates diversity

Bacteria are dynamic and present in large proportion in soil as reported by Colwell et al. (1985). Moreover, another reason for less frequency of total isolates could be targeted isolates under study i.e endophytes or ectophytes. Reasons for such bacterial presence and diversification behind mentioned above under our study could be soil environment like pH and temperature which further needed to be explored. Growth condition affecting bacterial community conducted by Robinson et al. (2016a) in wheat concluded bacterial composition is independent with growth stage, soil fertilizer treatment is always variable. A study carried out by Silicano et al. (1998) suggested that root morphologies in modern cultivars is responsible to affect the ability of microorganisms to absorb nutrients. Bacterial community assessment carried out by Germida and Siciliano (2001) in roots of *Triticum monococcum* and spring wheat *Triticum aestivum* concluded modern cultivar as more diverse compared to land race where two genera *Aureobacter* and *Salmonella* were detected more in modern cultivars. Similarly, Lilley et al. (1996) isolated 23 different genera with 114 isolates from *Beta vulgaris* further supporting endophytic communities that are independent between crop species. Sakurai et al. (2008) mentioned that amount of light and moisture conditions fluctuate root age changing microbial populations. Puri et al. (2018) reported diversification in bacteria is mainly due to root exudates, soil environment like pH and temperature.

### 4.2 Isolates obtained from root sample P and seed sample ds

Endophytes isolated from seed sample ds under our study manifested characters similar with root sample P in term of glucose fermentation. Firmicutes are dominantly reported in endosperm of several crops like rice, wheat and maize (Kaga et al., 2009; Ruiz et al., 2011) and few *Bacillus* species in spring wheat (Díaz Herrera et al., 2016). Endophytes present inside endosperm transmit generation after generation keep competitive advantage over beneficial endophytes (Kuźniar et al., 2020). Endophytic microbes are found to colonize at other parts of plant tissue like roots and leaves after germination (Herrera et al., 2016) keeping the chance to reach up to rhizosphere. Wheat roots are considered as best parts of higher endophytic diversity by Gdanetz, and Trail (2017) making rhizospheric zone as most probable niche of microbial diversity. McInroy and Kloepper (1995) also reported significant numbers of bacteria in the root interior surface than rhizoplanes who isolated numerous bacterial genera with altogether 1029 isolates from root of cotton and maize suggesting the plants have more diverse endophytic compared to other plants.

### 4.3 Isolates obtained from root sample R and root sample P

Germida et al. (1998) mentioned microbial communities and their dynamics in the rhizosphere are influenced by plants. Stressed plants generally release organic acids in the rhizosphere (Lopez brucio et al., 2000) attracting more endophytes around the root canopy. By difference in root exudation there exists bacterial diversity within cultivars of a particular species of wheat but remaining same in overall diversity was reported by Miller et al. (1989) Endophytes isolated under our study exhibited a wide range of characters from root sample P and root sample R in term of containing nitrogen fixing gene and glucose fermenting ability. Atkinson et al. (1975) also mention bacterial composition and diversity is significantly influenced by plant root exudation. One of the reason for diversification of endophytes in root sample obtained from seed of same variety could be due to root exudation due to stress of nutrients in soil collected from Kaski and Chitwan district (Hallmann et al., 1997) reported such bacterial compositions influences the development of phytopathogens and facilitate nutrient absorption from soil (Lynch, 1994). One of a reason for obtaining microbial diversity from root sample P and root sample R could be because of root exudation of plant having different physiology caused by varying nutrient status of soil samples. Fertilizer inputs affecting bacterial community is yet another strong reason of diversity as mentioned by (Seghers et al. 2004) which alters plant growth with respect to root exudation (Lopez Brucio et al., 2000). Hardoim et al. (2011) also mentioned in case of rice that plant genotype influences the endophytic bacterial community strongly with higher variation than soil type. DeBolt et al. (2015) reported that endophytic bacterial communities were less diverse in uncultivated lands as compared to farming lands. Many glucose fermenting containing isolates in root obtained from Rampur soil as compared to root sample obtained from Pokhara soil could be due to frequent application of organic manure in agronomy farm.

**4.4 Isolates obtained from root sample R and seed sample ds**

Under our study isolates obtained from root sample R and seed sample ds had nitrogen fixing containing genes but isolates varied in term of glucose fermentation. One of the reason for such diversity could be atmosphere received by seed and root sample. Review states bacteria population varies with the atmosphere it receives like as bacteria are more abundance in rhizospheres as compared to endophytic region (Khan & Doty, 2009). Germida and Siciliano (2001) reported some bacteria such as *Pseudomonas* were more abundant in roots of land race rather than modern cultivars.

**4.5 Glucose fermenting isolates**

Isolates under our study were having widely four different type of shapes namely imperfect circular, circular, wavy and circular. Puri et al. (2018) has also obtained isolates of 5 to 7 various shape ranges from sweet potato of various districts. Our result in term of glucose fermentation matches with study conducted by Nielsen et al. (2012) where it is reported that bacteria under phylum firmicutes were able to ferment glucose. Kong et al. (2018) also mentioned that most of monosaccharide like glucose fermenting bacteria was members of firmicutes. Shrivastava (2013) reported about 40% of isolates had glucose fermenting ability in his study. The isolate R1 has colony size of 1 to 2 mm and can ferment glucose. An experiment by Rodrigues et al. (2006) found the bacterial colonies of *Exiguobacterium sibiricum* are of same size and can ferment glucose. Moreover, Verma et al. (2016) isolated *Exiguobacterium sibiricum* as nitrogen fixing gene containing bacteria from wheat root in India bearing multiples growth promoting attributes on their experiment. But Verma et al. (2016) mentioned that *Exiguobacterium sibiricum* has colony color pink to red which is in contrast with our resulted colony color i.e. bright white. Moreover, further biochemical and morphological characteristics study is necessary to reach to near confirmation point.

Spank (1993) mentioned colony size of *Sutcliffiella cohnii* of 1-2 mm and colony color of creamy white. Gupta et al. (2020) mentioned *Schinkia azotoformans* is synonym with *Bacillus azotoformans*. Pichinoty et al. (1983) mentioned *Schinkia azotoformans* has negative indole production activity, negative citrate utilization activity, positive glucose fermentation and negative hydrogen sulfide production which are close about with present study. Jung et al. (2021) in his research mentioned *Lysinibacillus odyssesi* has negative indole production activity, negative citrate activity and negative hydrogen sulfide production activity. Additionally, Hayat et al. (2013) also mentioned *Lysinibacillus odyssesi* has colony size of diameter 1-2 mm negative indole production, negative hydrogen sulfide production and negative citrate utilization activity where in our result the isolate has negative citrate utilization activity, negative indole production activity and negative hydrogen sulfide production activity but colony size of diameter 2-3 mm. From the available information, *Lysinibacillus odyssesi* has more similar characteristics with isolate ds32. However, the biochemical properties obtained in our results are not sufficient to confirm the particular isolate as *Lysinibacillus odyssesi*. For confirmation the probable bacteria requires further research. Vaningelgem et al. (2006) mentioned *Entercocus thilandicus* ferments glucose at neutral media range around. (Wu et al., 2021) mentioned *Entercocus thilandicus* colony as dark red orange which is not in accordance with our result as we obtained the isolate as pale white color. Isolate ds24 in our result was found to have nitrogen fixing gene, glucose fermenting ability with pale white colony color. Moreover, Ahmed (2007) mentioned negative citrate utilization, negative hydrogen cyanide production and negative indole production with opaque unpigmented colony color for *Lysinibacillus sphaericus*. Biochemical character of *Lysinibacillus xylanilyticus* reported by Lee et al. (2010) to ferment sucrose and utilize citrate is not in accordance with present study. Similarly, circular shaped dark yellow colony color also is not in accordance with our findings. Shabamamol et al. (2018) reported nitrogen fixing

activity by *Lysinibacillus fusiformis* in rice, negative indole production test, negative citrate production and negative hydrogen cyanide test and white colony color. Jaganmai and Jinka (2020) reported *Lysinibacillus fusiformis* with negative hydrogen sulfide production and negative indole production test which is also in accordance with our result but positive citrate utilization activity is not in accordance with our findings. Two results in term of citrate utilization for same bacterial could be because they belong to different strains. However, only we cannot confirm the isolate certainly be *Lysinibacillus fusiformis* and further research is necessary to support particular bacteria.

Isolate ds6 had negative citrate utilization, negative hydrogen sulfide production, negative lactose and sucrose fermentation activity in present study. Miwa et al. (2009) mentioned the bacteria having colony color of pale white to greyish white as *Lysinibacillus parviboronicapiens* being close to our of pale white; diameter 2-3.5 mm and colony shape of wavy which is in accordance with present study of 2-3 mm and imperfect circular. *Lysinibacillus fusiformis* isolated by Verma et al. (2016) from wheat root in India was found to have multiples growth promoting attributes like nitrogen fixing genes. Characters obtained in present study on isolate ds6 suggests the probable bacteria to be however, we cannot confirm the particular bacteria with these characters. For near about confirmation, further study on characterization is necessary. According to our result of cel3 gene isolate ds6 has both targeted sized genes, but isolate ds24 wasn't found to have any targeted sized *cel3* gene. We can assume that isolate ds6 and isolate ds24 are different but for more confirmation further research is needed. Nielsen et al. (2012) used rRNA based SIP method to detect isolated microogranisms potential of fermenting glucose and highlighted bacteria of several genus under phylum firmicutes are good glucose fermenters. Bacterial isolates of genus like *Lactobacillus* having enzyme zymase were able to ferment glucose and produce butyric acid aerobically (Kong et al., 2008). Zaldivar et al. (2001) isolated bacteria can ferment glucose under alkaline conditions also. (Slobodkin et al. 2008) isolated glucose and glycerol fermenting isolates from deep soil environment. Akasaka et al. (2003) isolated several group of glucose fermenting isolates from rice field.

**4.6 Isolates with biochemical properties**

Verma (2001) states indole producing ability of bacterial isolates is widespread in soil and plant bacteria. Endophytes present inside plant parts like wheat can synthesize the phytohormone indole acetic acid, a native auxin synthesized by plants from animo acid, which is involved in plant stem and root growth regulation. Khan and Doty (2009) isolated about 30% bacteria from wheat which carry indole producing ability. Similarly, Puri et al. (2018) also isolated about 57% indole producing bacteria however we didn't obtain any isolates containing such beneficial character. It could be because of few numbers of roots and seed samples under our study. Bouffaud et al. (2016) and Rilling et al. (2018) also obtained few number of indole producing isolates from wheat which resemblances with abundancies of isolates under our result.
Similarly, the number of isolates capable of utilizing citrate from environment is comparatively low than results found by Van der Kooij (1977) where more than 90% isolates were capable of utilizing citrates. Shrivastava (2013) found 85% of endophytic isolates of rice with varying reaction ability were citrate utilizers and 38% of isolated bacteria from rice were able to ferment lactose. Study carried out by Germida et al. (1998) in the bacterial community suggested a lower Shannon-Weaver diversity index in field-grown plants than the community obtained from rhizoplane.

**4.7 Glucose fermenting isolates with *cel3* gene**

*Cel3* gene codes for protein under glycoside hydrolase family, has multiple functions like biological degradation activity of pant fiber components like cellulose and hemicellulose of cell wall (Bera et al., 2004). Cellulase gene favor the particular isolates to break down B-1,4-linkage of cell wall layer as mentioned in (Jayasekara & Ratnayake, 2019). Amongst total isolates of one hundred four under our study, 12 isolates were been found to have nitrogen fixing genes from root sample R while whereas six isolates been found from seed sample ds. Amongst eighteen isolates screened for presence of *cel3* gene, two isolates were found to have *nifH* gene and *cel3* gene with glucose fermenting ability, where four isolates with negative glucose fermentation activity had *cel3* gene of 400 bp. Further single isolate having many beneficial characters and with growth promoting traits or many isolates having few characters can be utilized in inoculation (Puri et al., 2018) though time of inoculation and the concentration of bacteria in the inoculum are significantly important in plant response to successful inoculation experiment (Bashan, 1985).

With the property of cellulase degrading enzyme bacteria move from root interior to exterior region and this movement is largely regulated by plants suggesting the bacterial community is variable with time (Germida & Siciliano (2001). One of the reasons behind the difference in frequency is assigned total number of root and whole seed samples. This fact is also supported by Saha, et al. (2006) who mentioned microorganisms having cellulase genes can break cell wall layer and have in and out mechanisms. Reasons behind the presence of nitrogen fixing gene isolates in seed sample ds but not in root sample P could be because of the cellulase activity of isolates that can enter and exit the plant cell. Puri et al. (2018) in his study also mentioned around 70% endophytic bacteria isolated had medium to strong cellulase activity having potential for plant cell wall breakdown.Dominating role in determining the bacterial community composition is played by plants providing additional selection pressure explaining lower diversity and greater evenness in endophytes than rhizospheric community (Chanway, 1996; Germida et al., 2001).

## 5. Conclusion

The root sample P had high number of citrate utilizing isolates whereas root sample R had high number of *nifH* gene containing isolates under study. Glucose fermenting isolates containing *nifH* gene were found to be R1, ds6, ds7, ds22, ds24 and ds32. Glucose fermenting isolates having *cel3* gene were obtained to be ds6 and ds7 which keep endophytic ability along with growth promoting ability. Further research scope exists to use these microbes as a bio fertilizer in plant growth promotion study by inoculation.

## Statements and Declarations

The authors declare that there is no conflict of interest regarding the publication of this article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## Competing Interests

The authors have no relevant financial or non-financial interests to disclose

## Data Avaibality

Data will be made available on request.

## Author Contribution

Conceptualization: Subash Devkota and Ramesh Raj Puri; Data curation: Subash Devkota; Investigation: Ramesh Raj Puri, Subash Devkota, and Himal Luitel; Methodology: Subash Devkota and Ramesh Raj Puri; Resources: Subash Devkota; Software: Subash Devkota and Pankaj Kumar Yadav; Supervision: Ramesh Raj Puri, and Himal Luitel; Validation: Ramesh Raj Puri ; Visualization: Subash Devkota, Pankaj Kumar Yadav, Sushil Bhandari, and Santosh Rasaily; Writing , review & editing: Subah Devkota and Pankaj Kumar Yadav

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