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Production of biofuel from sugarcane molasses by diazotrophic *Bacillus* and recycle of spent bacterial biomass as biofertilizer inoculants for oil crops

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ABSTRACT

This study was designed to identify the most potent N_2-fixing and biofuel producing *Bacillus* species. Four isolates were selected as the most efficient N_2-fixing organisms which confirmed by *nifH* gene expression. These isolates were identified genetically by 16S rRNA as *Bacillus thuringensis*, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis*. The highest biohydrogen production was 2450 and 2300 ml/L from 6% sugarcane molasses by *B. thuringensis* and *B. subtilis* respectively. Nitrogenase activity of *B. thuringensis* and *B. subtilis* were 1.4 and 1.3 µmol C_2H_4 min^{-1} mg protein $^{-1}$ at 6% molasses. Ethanol production was 1.55 and 1.03 g/L, while butyric acid was 10.39 and 5.9 g/L at 6% molasses by *B. thuringensis* and *B. subtilis* at 6% molasses, respectively. Acetic acid formation was 1.1 and 0.55, lactic acid was 0.07 and 0.05, while butyric acid was 10.39 and 5.9 g/L at 6% molasses by *B. thuringensis* and *B. subtilis*, respectively. Spent bacterial biomass of the two *Bacillus* species were reused as a biofertilizer for enhancing the growth of sunflower and corn plants. Inoculation of sunflower and corn seeds with *B thuringensis* and *B subtilis* significantly increased dry weight, total protein, total carbohydrates and pigment contents over control plants. This enhancement could be attributed to the efficiency of biological N_2-fixation due to nitrogenase activity of the tested *Bacillus* species. These results suggest that the possibility of interlinking biofuel technology with biofertilizer production by reusing N_2-fixing spent bacterial biomass of *Bacillus* could be increase the economic feasibility of the bioenergy production from molasses.

1. Introduction

Energy plays an essential role in today's modern society (Grafton et al., 2015). The modern world depends massively on the use of energy obtained from fossil fuels. Fossil fuel is the primary source for food production. The green revolution is mainly relying on fossil resources. Nearly 80% of the world's fossil energy utilized in the manufacture of synthetic fertilizers, pesticides, irrigation, and machines (FAO, 2011). Global utilization of nitrogen fertilizer has been raised more than six-fold over the past 40 years (Tilman, 1999). Manufacture of nitrogen fertilizer utilized more than 50% of the total energy applied to the agriculture sector (Woods et al., 2010). Agrochemicals production especially nitrogen using the Haber–Bosch Process, consume massive amounts of fossil energy, releasing about 465 tera-grams carbon dioxide into the atmosphere each year (IFA, 2009). It has been estimated that 30% of total fossil fuel is consumed for manufacture of nitrogen fertilizer used in maize production (Tilman, 1999). The nitrogen fertilizer industry is responsible for up to 1.2% of all anthropogenic greenhouse gas emissions (Wood and Cowie, 2004; Taheripour et al., 2017).

Nitrogen fertilizer application could induce further emissions. The process of nitrification and de-nitrification of organic and mineral nitrogen compounds leads to the release of huge amounts of nitrous oxide from soils (Snyder et al., 2009). Nitrous oxide emission from agro-ecosystem has been calculated approximately 1.5% of total anthropogenic greenhouse gas emissions (IPCC, 2006; IFA, 2009).

Modern agriculture consumes high energy crop production technology, especially with respect to nitrogen fertilizers and pesticides. More critical problems are expected when there is a world fossil energy shortage (Gellings, 2009). To minimize energy utilization and greenhouse gas emission, modern agriculture might employ such alternatives as biofuels, biofertilizers, and biopesticides to reduce the high energy

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demand of chemical fertilizers and pesticides (Aggani, 2013; Chen et al., 2018). The most important challenge forefront sustainable energy and environmental issue is to develop processes for energy production that are eco-friendly and sustainable. Obviously, energy production of biological sources such as economic crops as an alternative a choice is not a sustainable manner. Therefore, more efficient and sustainable routes of energy generation are required.

Microorganisms, including eubacteria and fungi, are versatile and have the capacity to produce fuel and fuel precursors from agro-industrial wastes (Montero-Rodríguez et al., 2016; Leiva-Candia, 2014; Gad El-Rab et al., 2018). Some species of genus *Bacillus* have the ability to ferment a wide range of organic wastes. These species show enormous promise to concurrently create biofuels, biofertilizers, and biocontrol. Non-symbiotic N_2-fixation has been well documented to be particularly carried out by some members of the eubacteria where atmospheric N_2 is converted to NH_3 and catalyzed by bacterial enzyme nitrogenase. *Bacillus spp*. are spore former facultative anaerobic Gram-positive bacteria present in the soil, water, and plant surfaces. These bacteria can adjust its metabolic activity and respiration depending upon the oxygen availability and fix atmospheric N and produce H_2 under anaerobic conditions (Porwal et al., 2008; Van and Abeer, 2009; Patel et al., 2011). Some species of genus *Bacillus* having the capacity to fix atmospheric N_2 can be used as efficient biofertilizer to improve the yield of commercial crops (Radhakrishnan et al., 2017). Also, spores of *Bacillus thuringiensis* have been widely applied as a biopesticide to control insect pests that damage agricultural crops (Sarker and Mahbub, 2012; De Maagd, 2015).

In recent years, molasses become the ideal and sustainable substrate for bacterial fermentation. Molasses has some economic advantages such as being relatively cheap and suitable to operate as a liquid, with no need for hydrolysis processes before using. The availability of molasses is universal and mainly depends on the manufacture of sugar from sugarcane and sugar beets. Sugarcane is massively cultivated in the tropical and sub-tropical regions such as Africa, Asia, Australia, Central, and South America. Sugar beets are grown mainly in North America, Europe, Northern Africa as well as Northern Japan and China. Approximately 80% of the sugar manufacture is produced from sugarcane. The world molasses production is approximately 50 million tons and about 15% of the total molasses is beet molasses (Arifeen, 2014). The most sugarcane molasses suppliers are Sudan, Pakistan, Thailand, and Indonesia. In Egypt, approximately 370 thousand tons of molasses are produced annually as a by-product during the sugar industry processes. Molasses produced from the sugar mills is a very valuable co-product because it has a high sugar content making it a promising substrate in fermentation processes (Whiteman and Gueguim-Kana, 2014; Bagy et al., 2014; Abd-Alla et al., 2017).

The main obstacle of the mass biofuels generation is the high cost of the production. The most important way that can substantially reduce the cost of biofuels fermentation and increase the economic feasibility of biofuel production is to reuse the spent bacterial biomass as biofertilizer and biocontrol.

This study aims to introduce an integrative approach for the concurrent production of extracellular metabolites of high energy fuels (hydrogen and ethanol) with valuable secondary chemical compounds of butyric acid, lactic acid and acetic acid by *Bacillus* species from sugarcane molasses. The objective was extended to reuse the spent bacterial biomass as biofertilizer inoculants to improve the growth of maize and sunflower plants (oil crops). This integrated system may become of considerable importance and the commercially valuable approach for reduction of the production costs of biofertilizer through the concomitant production of biofuel in one pot fermentation process.

2. Materials and methods

2.1. Sample collection and bacterial isolation

Domestic wastewater and soil samples were collected from Assiut Governorate, Egypt. One grams of soil samples (one ml of water samples) was suspended in 99 ml of sterile distilled water and shaken vigorously for 2 min. The samples were heated at 70°C for 60 min in a water bath to inactivate vegetative cells. Then the soil suspensions were kept for 2 h at room temperature to precipitate soil particles. 100 µl of each sample was plated on NA medium (Oxoid), using streak method of inoculation. The plates were incubated aerobically at 37°C for 24–48 h. Total of twenty-one colonies were chosen based on their morphology. White to off-white colonies were selected and categorized as possible *Bacillus* colonies. Colonies with different morphological appearance were sub-cultured onto fresh NA for the purpose of identification and further study.

2.2. Phenotypic characterization and identification of Bacillus

Pure cultures were characterized by physiological and biochemical tests as described in Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005) such as Gram stain, shape, indole production, catalase test, cellulose hydrolysis, starch hydrolysis, Voges-Proskauer reaction, utilization of carbohydrates, H_2S production and gas production.

2.3. Nitrogenase activity (EC 1.18.6.1) assay

Twenty-one bacterial isolates were examined for their ability to fix atmospheric nitrogen (nitrogenase activity) by acetylene reduction using Thermo Scientific TRACE GC Ultra (Abd-Alla et al., 2019). Nitrogen fixation of the isolates was determined in Burk's N-free medium by the acetylene reduction assay (Hardy et al., 1968). Burk's N-free medium comprising (g/L): glucose 10; KH_2PO_4 0.41; K_2HPO_4 0.52; Na_2SO_4 0.05; $CaCl_2$ 0.2; $MgSO_4{\cdot}7H_2O$ 0.1; $FeSO_4{\cdot}7H_2O$ 0.005; $Na_2MoO_4{\cdot}2H_2O$ 0.0025; agar 1.8 (Wilson and Knight, 1952; Parka et al., 2005). Nitrogenase activity is defined as µmoles of ethylene per mg of protein, cellular protein was estimated using egg albumin as the standard protein according to Lowery method (Lowry et al., 1951).

2.4. Identification of Bacillus isolates using partial 16S rRNA gene sequencing

The most potent N_2-fixing isolates were identified using universal primers; 27F: (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R: (5′ GGTTACCTTGTTACGACTT-3′) in amplification (Lane, 1991), reactions were prepared in a total volume of 50 µl reaction; 25 µl GoTaq® DNA polymerase master mix (Promega, USA), 2 µl primer F, 2 µl primer R, DNA template and nuclease-free water, according to the thermal profile 95°C 3 min, 95°C 1 min, 50°C 1 min, 72°C 1.5 min for 40 cycles followed by 72°C 10 min in Biometra Thermal cycle, and the results visualized in 1% agarose gel stained with ethidium bromide under UV light, the PCR product was purified using QIAquick PCR purification kit (Qiagen) and sequence reaction was performed using Bigdye terminator sequencing kit (Applied Biosystems) by sanger technique then loaded on the sequencer instrument (ABI prism 310 genetic analyzer, Applied Biosystems). BLASTN searches were done using the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and phylogenetic tree was constructed by 16S rRNA gene sequences from different standard bacterial strains obtained from GenBank using clustalW version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html).

2.5. Detection of nifH gene

RNA was extracted using QIAamp RNA extraction kit (Qiagen), using the *nifH* Primer; F: (5′-GGCTGCGATCCGAAGGCCGA-3′) and R: (5′-GCGTAGAGCGCCATCATCTC-3′) (Bürgmann et al., 2004). RT-PCR was prepared in a total volume of 25 µl with the following conditions: 5 µl buffer, 1 µl dNTPs, 1 µl enzyme, 1 µl primer F, 1 µl primer R, RNA template and nuclease-free water using one-step RT-PCR kit (Qiagen), according to the thermal profile 50°C 30 min, 95°C 5 min, 95°C 40 s, 50°C 40 s, 72°C 1 min for 40 cycles followed by 72°C 10 min in Biometra Thermal cycle, PCR products were visualized in 1% agarose gel stained with ethidium bromide under UV light, the *nifH* gene product is approximately 370 bp. The positive PCR product was purified using QIAquick PCR purification kit (Qiagen), sequence reaction was set up using Bigdye terminator sequencing kit (Applied Biosystems) by sanger technique then loaded on the sequencer instrument (ABI prism 310 genetic analyzer, Applied Biosystems).

2.6. Gene expression

Semi-quantitative PCR used to show *nifH* gene expression for the most active N_2-fixing *Bacillus* isolates, RT-PCR reaction was performed, and all the parameters and conditions used in the experiment were kept constant using equal concentration of RNA which estimated by DNA/RNA spectrophotometer (GeneQuant 1300 spectrophotometer). The PCR products containing the specific band of 370 bp was detected by Bio doc Analyzer software (Biometra, Germany) using 100 bp DNA–ladder (iNtRON Biotechnology) with well-known bands intensity according to the ladder manual (Cat no. 24073). The expression of each sample was estimated by comparing the *nifH* gene bands intensity of the tested *Bacillus* samples to the bands of the marker (Antiabong et al., 2016) and the intensity was calculated by the analyzer program.

2.7. Nucleotide sequence accession numbers

The nucleotide sequences of the isolates *Bacillus thuringensis*, *Bacillus subtilis, Bacillus pumilus*, *Bacillus licheniformis* were deposited in the GenBank nucleotide sequence database under GenBank accession nos. **MG744606, MG738313, MG738314, MG738312**. The nucleotide sequence of the *nifH* gene of *Bacillus thuringensis* was deposited in the GenBank database under the GenBank accession no. **MH141502.**

2.8. Selection of hydrogen-producing Bacillus strains from sucrose and sugarcane molasses substrates

The most active N_2-fixer strains were tested for their ability to produce hydrogen. Sucrose medium was used for the bacterial cultivation, maintenance, and hydrogen production. The sucrose medium contained (g/L): sucrose 15 g; tryptone 5 g; K_2HPO_4 14 g; KH_2PO_4 6 g; $(NH_4)_2SO_4$ 2 g and $MgSO_4$ .7 H_2O 0.2 g. 70 mL of precultured sucrose broth (OD_{660} = 0.2) of each bacterial strain was injected into glass bottles containing 630 mL of sucrose medium. The higher hydrogen producer strains on sucrose were selected for hydrogen production from sugarcane molasses.

Sugarcane molasses was obtained from Sugar Technology Research Institute, Assiut University, Assiut, Egypt; and kept in the refrigerator until used. Some physical and chemical characteristics of sugarcane molasses were determined as follows: (%) ash 12.50 ± 0.5, total sugar 56.50 ± 1.0, non-fermentable sugar 4 ± 0.2, fermentable sugar 51.5 ± 0.1, reducing sugar 24.52 ± 0.1, nitrogen 0.6 ± 0.1, protein 4.12 ± 0.1, Cao 1.5 ± 0.1, P_2O_5 0.3 ± 0.01, SO_4 g/L18 ± 2.0 and pH 5.1 ± 0.1.

Sugarcane molasses was used as a substrate for hydrogen production at different concentrations (40, 60 and 80 g/L). The initial pH of the culture medium was adjusted to 7. The sugar concentration was estimated before and after culture fermentation by the anthrone-sulfuric method (Fales, 1951). The H_2 was analyzed according to Abd-Alla et al. (2019). Samples from the medium were withdrawn from the replicated fermented bottles to determine; nitrogenase activity by the acetylene reduction technique (Abd-Alla et al., 2019).

The bacterial growth response was assessed by following the OD at 660 nm using Thermo scientific double beam spectrophotometer (Evolution 160, UV-VIS, Germany), total cellular protein, final pH value and residual sugar content (Halhoul and Kleinberg, 1972). A Control fermentation of the two tested isolates under anaerobic conditions using sucrose medium was conducted.

2.9. Production of ethanol and organic acids from molasses by Bacillus strains

Under anaerobic conditions at 37°C, the concentration of ethanol, acetic, lactic and butyric acids were evaluated in fermentation media using a gas chromatography (Trace GC ultra) (Abd-Alla and El-enany 2012).

2.10. Preparation of spent bacterial biomass to be used as biofertilizer inoculant

A spent fermentation medium was collected into a 50 ml tube and centrifuged at 3000 rpm for 10 min. The pellet containing bacterial cell biomass was washed, re-suspended in 25 ml of 0.1 M sterile potassium-phosphate buffer (pH 7.4). The bacterial suspension will be used as inoculant.

2.11. Plant growth and bacterial inoculation

The experiment was carried out in a wire proof greenhouse, 2-kg pots were filled with sterilized soil, and the sterilization was carried out by autoclaving the soil twice at 121°C for 2 h. Some physical and chemical characteristics of the experimental soil were determined as follows: pH 7.8, clay 51.41%, sand 19.5%, silt 28.3%, cations (mg/100 g soil); Na^+ 0.5, K^+ 0.57, Ca^{++} 0.25, Mg^{++} 0.39 and anions (mg/100 g soil); Cl^- 1.43, HCO^{-3} 0.7.

The experiment included two economic oil crop plants namely, *Helianthus annuus L.* Giza 102 (sunflower) and *Zea mays L.* single cross Giza 2 (corn). Seeds were obtained from the Agronomy Department, Faculty of Agriculture, Assiut University, Egypt. Seeds were sterilized by exposure to a sodium hypochlorite solution (5%) for 2 min, washed in 70% ethanol for 2 min then washed with distilled water. Seeds were soaked in bacterial suspension (1×10^{7} CFU) of *B. thuringensis* and *B. subtilis* for 1 h then sown about 1 cm depth in pots. Control seeds were soaked in the same buffer (0.1 M sterile potassium-phosphate buffer, pH 7.4) and pots received 320 mg N as NH_4NO_3 before sowing for comparison between chemical nitrogen fertilizer and biofertilizer. Three replicates of each treatment were set up for both plants.

Seven seeds were planted per pot. After seven days of germination, seedlings were selected upon similarity in height, a number of healthy leaves then thinned to three plants per pot. Plants were irrigated until harvesting with tap water for a period of 50 days (November–December) and (March–April) for sunflower and corn, respectively. Plants were harvested after 50 days; shoots and roots were carefully collected and washed under running water. Fresh weight, dry weight after drying in an oven at 70° C for 72 h were estimated. Dried shoots and roots were ground to a fine powder and were analyzed for the leaf chlorophyll content (Lichtenthaler, 1987), total protein (Lowry et al., 1951) and total carbohydrates (Fales, 1951) in both shoots and roots.

2.12. Statistical analysis

The data were subjected to one-way ANOVA using the SPSS 19.0 software program. The Experiments were repeated three times with three independent replicates. Means and standard errors were calculated for three replicates. Means were compared by Duncan's multiple range tests and statistical significance was determined at 5% level.

3. Results and discussion

3.1. Isolation and identification of the bacterial isolates

Twenty-one bacterial isolates were recovered from wastewater and soil on a nutrient agar medium and showed white to off-white colonies then identified by the biochemical tests, all isolates were Gram-positive rod cells with spore formation. They were positive for gas production, voges-Proskauer, catalase test, starch hydrolysis, cellulose hydrolysis, nitrogenase activity and carbohydrates utilization) glucose, sucrose, lactose and mannitol) but not indole production, methyl red and H_2S production.

3.2. Nitrogenase activity

Out of these twenty-one isolates, twenty isolates showed positive results with variation in nitrogenase activity and one isolate was negative. The most potent N_2-fixing isolates (4 isolates) were chosen for further study.

3.3. Identification using partial 16S rRNA gene sequencing

These four isolates were identified using 16S rRNA gene sequences. The partial 16S rRNA gene sequence of the isolate 1 had sequences of 98% similarity with *Bacillus licheniformis* (MF470191) respectively, isolate 2 shared 98% similarity with *Bacillus subtilis* (KY203663) as supported by the phylogenetic tree (Fig. 1). 16S rRNA genes of the isolate 3 showed 98% similarity to that of *Bacillus pumilus* (KP192031). The 16S rRNA gene sequence from the analyzed isolate 4 shared 98% similarity with *Bacillus thuringensis* (KX057531). The phylogenetic tree was inferred from a multiple sequence alignment of 16S rRNA sequence data by the neighbor-joining method.

3.4. Identification of nifH gene

Nitrogenase enzyme was confirmed by the *nifH* gene as a marker for biological N_2-fixation (Poly et al., 2001; Wartiainen et al., 2008). The tested isolates were positive for *nifH* gene amplification. The PCR products showed clear band at 370 bp on an agarose gel, while negative control didn't generate PCR product of *nifH* gene. These results revealed no DNA contamination and subsequently confirmed that the RT-PCR products were from RNA samples (Fig. 2A). The length of the *nifH* gene product from different isolates was similar to previous reporting by using the same primer (Bürgmann et al., 2004).

3.5. Detection of nifH gene expression

By optimized the concentration of all sample's RNA to 5 ng/µl and applied equal amount in each tube. The intensities of RT-PCR products on agarose gels were 114, 92, 76, 35 ng/µl for *B. thuringensis*

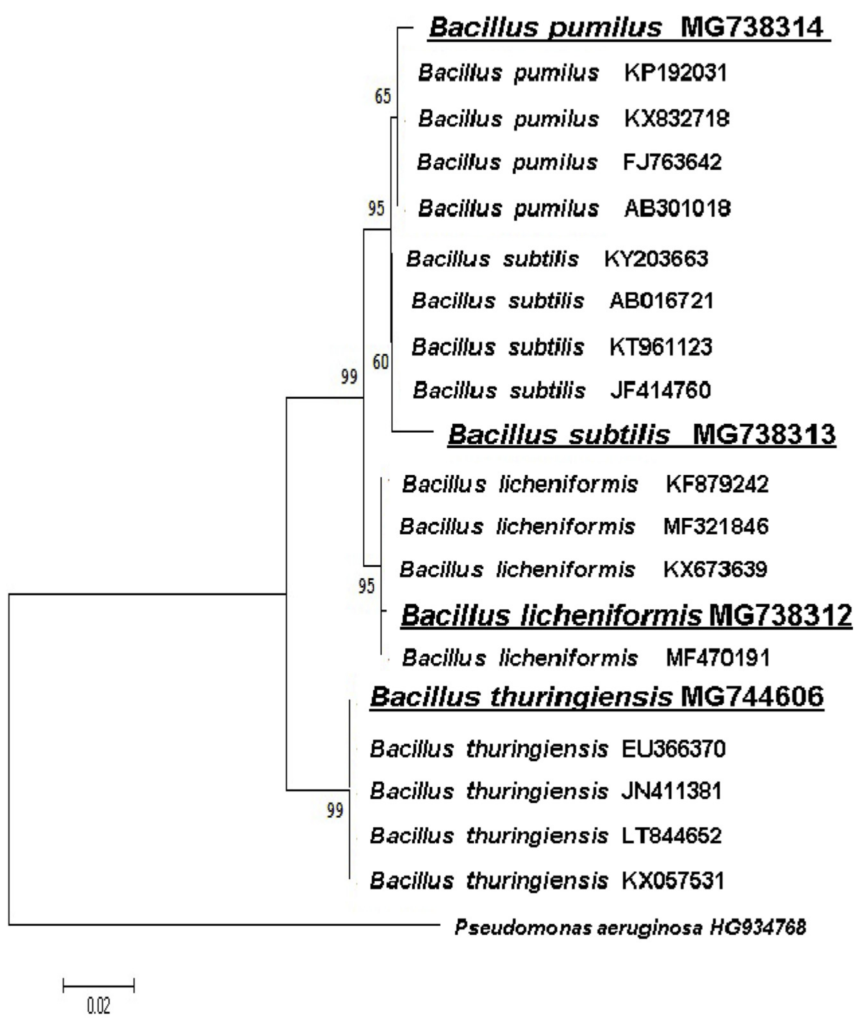

Fig. 1. Phylogenetic tree indicating the phylogenetic relationship of the isolated strains. Isolates are indicated in bold with accession number. A neighbor-joining tree was calculated using partial 16S rRNA gene sequence. *Pseudomonas aeruginosa (*HG934768) was used as outgroup. Numbers within the dendrogram indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees. Bar, 2% sequence divergence.

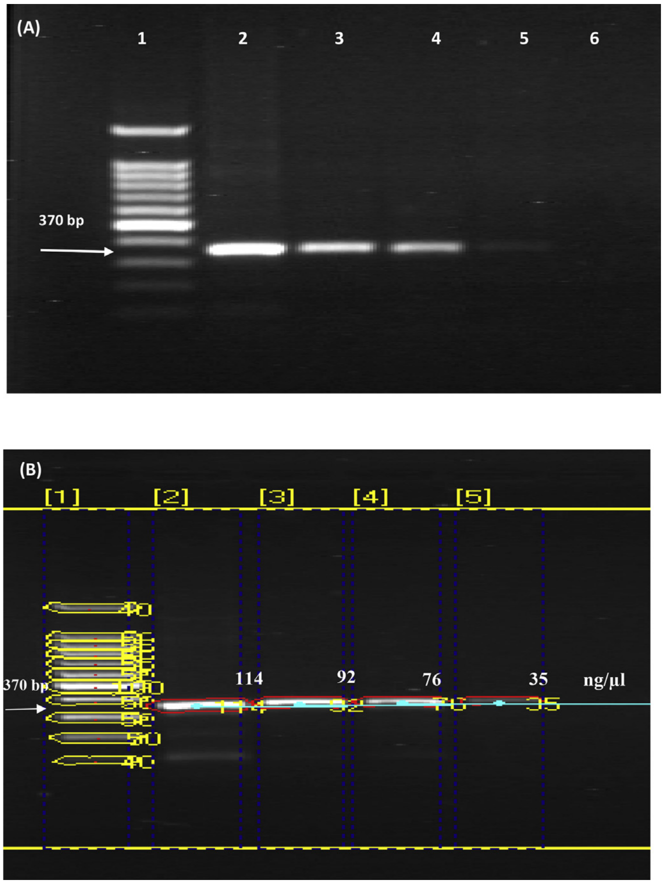

Fig. 2. **(A)** Agarose gel analysis of *nifH* gene (370 bp) from different nitrogen-fixing *Bacillus* strains amplified by RT-PCR using total RNA extracted. Lane, (1) DNA marker 100 bp, (2): *B. thuringiensis*, (3) *B. subtilis*, (4) *B. pumilus*, (5) *B. licheniformis* and (6) negative control. **(B)** Semi-quantitative expression of *Bacillus* strains *nifH* gene using BioDoc analyzer program software; showing the intensity differences between PCR bands (370 bp encoded *nifH* gene). Lane (1): the DNA marker 100 bp, Lane (2): *Bacillus thuringiensis*, Lane (3) *Bacillus subtilis*, Lane (4) *Bacillus pumilus* and Lane (5) *Bacillus licheniformis*.

(MG744606)*,* *B. subtilis* (MG738313), *B. pumilus* (MG738314) and *B. licheniformis,* respectively. The difference in bands (*nifH* gene) intensity confirming that there was a different N_2-fixation capacity between the tested strains (Fig. 2B). The intensity of *nifH* gene expression was a good indicator for N_2-fixation abilities and may be used for screening N_2-fixing potentiality of the isolates (Akter et al., 2013). Direct quantification of band intensities on gels is commonly used as a simple procedure for quantification of DNA template concentrations (Bogan et al., 1996; Garcia et al., 2001; Widmer et al., 1996). This positive connection between *nifH* gene expression and N_2-fixing potentiality means that quantification of the expression of this gene may be used to screen the bacterial isolates for N_2-fixing and hydrogen-producing capacity.

3.6. *Biofuel production by Bacillus strains from sucrose and molasses*

These most potent N_2-fixing strains were tested for their capability to produce hydrogen; when sucrose was used as the carbon source, *B. thuringensis* was the highest hydrogen-producing strain (1400 mL) followed by *B. subtilis* (1200 mL), *B. pumilus* (900 mL) and *B. licheniformis* (800 mL) (Fig. 3). The maximum H_2 formation rate was produced by *B. thuringensis* MG744606 (19.44 mL H_2/h) followed by *B. subtilis* MG738313 (16.66 mL H_2/h), *B. pumilus* MG738314 (12.5 mL H_2/h) and *B. licheniformis* MG738312 (11.11 mL H_2/h). *Bacillus thuringensis* and *Bacillus subtilis* produced H_2 gas with an efficiency of 1.16 and 1.06 mol H_2/mol sucrose, respectively. It was reported that *Bacillus* species have

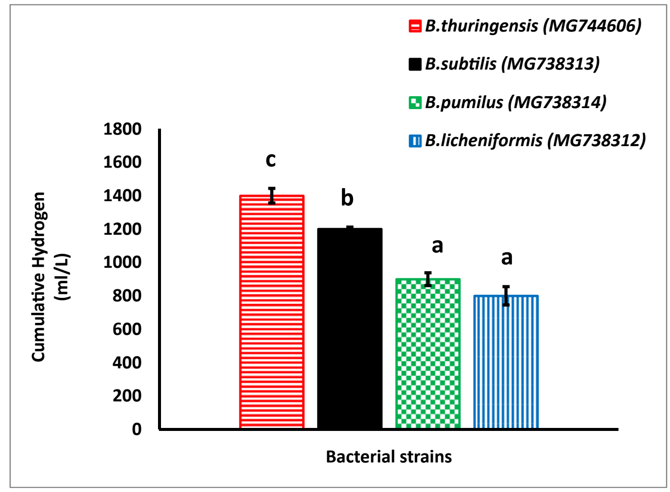

Fig. 3. Cumulative hydrogen production from sucrose by different *Bacillus* strains. Values represent the mean of three replicates ± standard error (vertical bars) within each isolate. Means with the same letter are not significantly different among bacterial isolates at the 0.05 level using Duncan's multiple range tests.

the capacity to produced 1.53 mol/mol hexose when grown on sucrose medium (Sung et al., 2002). It was reported that native hydrogen-producing bacteria have the potentiality to produce 3.61–4.46 mol H_2/mol hexose when grown at 25°C from sugary wastes (Perera and Nirmalakhandan, 2010). Various bacterial species have been reported to produce hydrogen in the range of 1.5–1.65 mol/mol glucose (Patel et al., 2010; Kumar et al., 2013) and 1.634 mol H_2/mol hexose by *Bacillus* (Glinwong et al., 2017).

Some *Bacillus* isolates can produce H_2 using biodegradable substrates as a source of carbohydrates in a range of 0.42–2.60 mol/mol hexose (Jeong et al., 2008; Liu and Wang, 2012; Kotay and Das, 2007, 2009; Song et al., 2013; Patel et al., 2011). *Bacillus* grows well and can produce biohydrogen under anaerobiosis (Porwal et al., 2008), and considered as strong candidates for biohydrogen production due to (i) they can survive and tolerate the drastic environmental conditions, (ii) they have enormous enzymatic activities that able to utilize wide range of agro-industrial waste as sustainable substrate for hydrogen generation, (iii) they do not need light for hydrogen fermentation, (iv) Endospore-forming *Bacillus* are being used as biofertilizer, biocontrol and probiotics, hence, they may not impose environmental health problems (Schallmey et al., 2004; Kumar et al., 2013).

Currently, many fermentable substrates are utilized for biofuel production such as sugarcane molasses (Hassan et al., 2015). Sugarcane molasses contains high fermentative carbohydrate content (≥55% sucrose) that used as the carbon source for bacterial growth and fermentation process (Abd-Alla et al., 2014; Tugba and Patrick, 2012; Tunçay et al., 2017). Gram-positive bacteria such as *Bacillus* spp. characterized by their capacities to withstand relatively low pH, high temperature, high sugar, and various other harsh conditions, which could be used to develop an advanced biocatalyst and improve the commercial competitiveness of biofuel production (Dien et al., 2003; Romero et al., 2007). *Bacillus thuringensis* (MG744606) and *Bacillus subtilis* (MG738313) the highest hydrogen-producing strains from sucrose were tested for hydrogen, bioethanol and organic acids production from sugarcane molasses as a substrate. The price and accessibility of sugarcane molasses make it an attractive feedstock for biohydrogen production in many countries (Bagy et al., 2014; Quan et al., 2005; Kumar et al., 2018).

The growth pattern at 660 nm (OD_{660}) and protein content of the two tested isolates were determined on sucrose and sugarcane molasses (40, 60 and 80 g/L) (Fig. 4). The maximum fermentative H_2 production of 2450 and 2300 mL/L was obtained from 6% molasses compared with

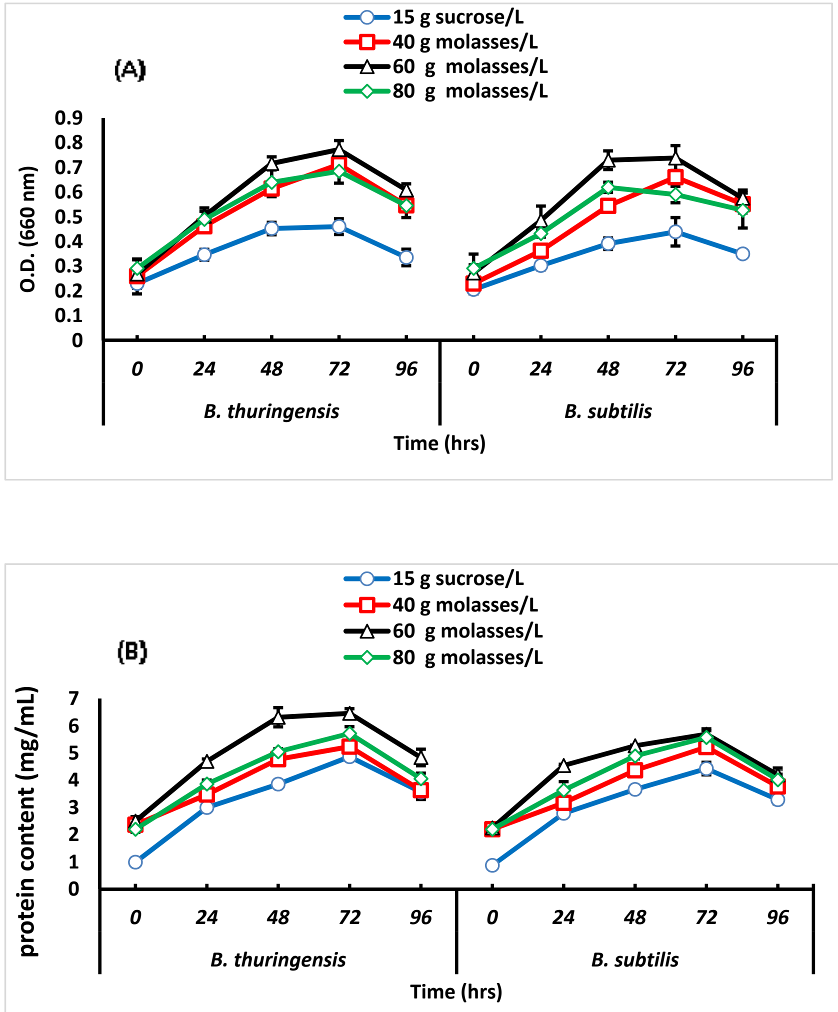

Fig. 4. (A) Growth curve and **(B)** protein content of *Bacillus thuringiensis* and *Bacillus subtilis* grown on sugarcane molasses. Values represent means of three replicates ± standard error (vertical bars).

control 1400 and 1200 mL by B. *thuringensis* (MG744606) and *B. subtilis* (MG738313); respectively. (Fig. 5A). Hydrogen efficiency of *B. thuringensis* and *B. subtilis* were 1.37 and 1.27 mol H_2/mol sucrose; respectively released from 6% molasses comparing with control medium (Table 1). Argun et al. (2017) reported that hydrogen yield was (202.32 mL H_2/g COD), with rate (14.02 mL H_2/h) and H_2 percentage (41.83) at an optimum temperature from hot spring microflora. Molasses is considered as a renewable source that can be converted into biohydrogen by dark fermentation due to its high organic content (Wu et al., 2013).

Also, the maximum ethanol production was achieved from 6% molasses as 1.55 and 1.031 g/L whereas in sucrose medium was 0.517 and 0.465 g/L by *B. thuringensis* and *B. subtilis*; respectively (Table 1). Such amount of ethanol is minuscule and required more optimization to enhance the production. To decrease the dependence on fossil fuel, the worldwide have been stimulated researcher's efforts for the production of ethanol from lignocellulosic biomass (Kataria and Ghosh, 2011). It was reported that *B. subtilis* have the capacity to ferment banana and orange peels as a substrate for bioethanol production (Singh, 2014). Optimization the conditions of ethanol fermentation of molasses and food waste leachate is a vital factor such as temperature, dilution rate and sugar concentration for higher production (Perego et al., 1985; Le Man et al., 2010).

Bacillus thuringensis and *Bacillus subtilis* examined for their ability to produce organic acid as acetic, lactic and butyric acids. Acetic acid production was 0.07 and 0.06 g/L by *B. thuringensis* and *B. subtilis* which increased to 1.1 and 0.55 g/L when grown on 6% molasses; respectively while lactic acid was 0.02 and 0.01 g/L increased to 0.07 and 0.05 g/L at 6% molasses by *B. thuringensis* and *B. subtilis*; respectively. Butyric acid was more than the other acids showed high values at 4.23 and 4.07 g/L increased to 10.39 and 5.9 g/L respectively by *B. thuringensis* and *B. subtilis* on 6% molasses at pH 4.5 (Table 1). After fermentation, the final pH was sharply decreased due to the presence of acetic, lactic and butyric acids generated from glucose by *Bacillus subtilis*. In similar studies, it was documented that lactate, acetate, acetoin, ethanol, and succinate were the main fermentation products of *B. subtilis* (Speck and Freese, 1973; Nakano et al., 1997). The accumulation of organic acids regarded as the decrease in pH, leading to a significant fall in hydrogen production (Kumar and Das, 2000; Ginkel and Logan, 2005). The butyric acid was the main metabolite with a partial contribution of ethanol (Chojnacka et al., 2011).

Nitrogenase activity is the main source of hydrogen gas evolution as a byproduct of N_2-fixation (Dietrich et al., 1974; Potrikus and Breznak, 1977). N_2-fixing abilities estimated by acetylene reduction activity showed that *B. thuringensis* was highest nitrogenase activity (1.4 µmol C_2H_4 min^{-1} mg^{-1} protein) followed *by B. subtilis* (1.3 µmol C_2H_4 min^{-1} mg^{-1} protein) when grown on 6% molasses compared with sucrose medium (Fig. 5B). It was reported that the acetylene reduction activity by *B. pumilus* and *B. subtilis* were 437.26 and 418.45 nmole C_2H_4 h^{-1} mg^{-1} protein, respectively (Kaushal and Kaushal, 2015).

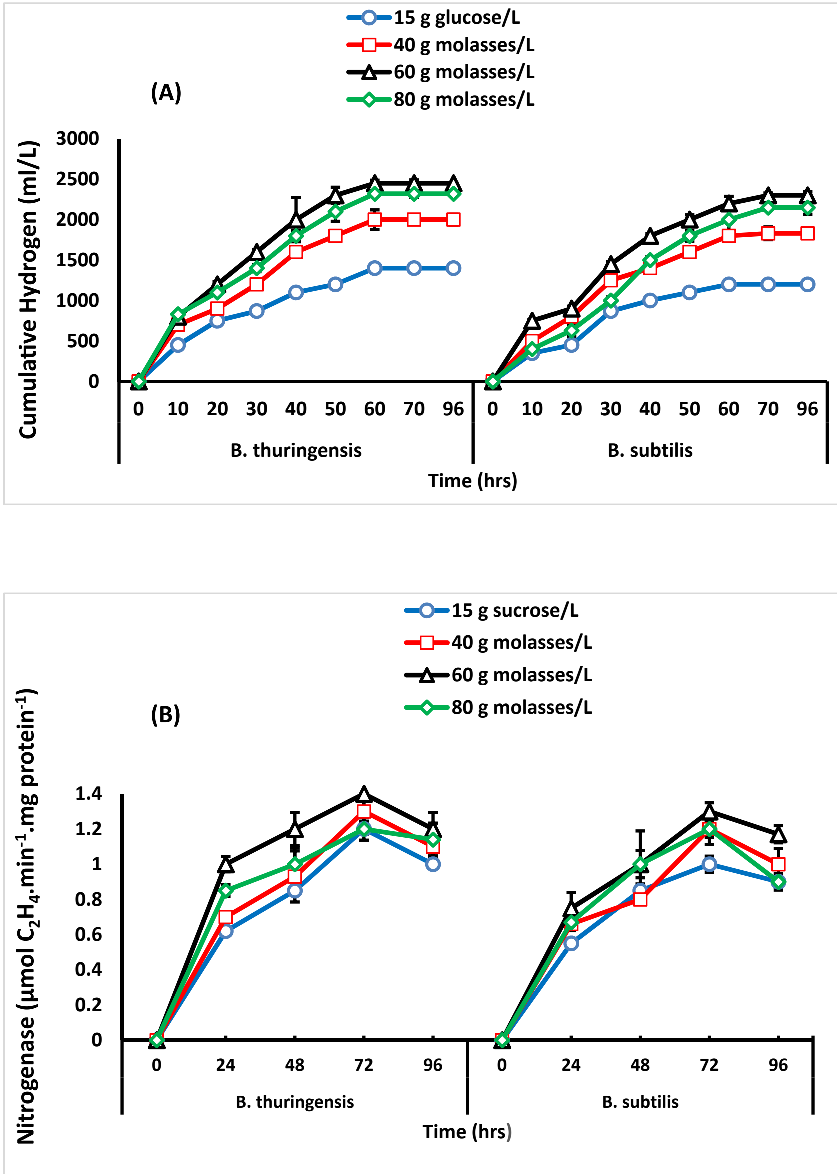

Fig. 5. (A) Cumulative hydrogen production and **(B)** nitrogenase activity of *Bacillus thuringiensis* and *Bacillus subtilis* grown on sugarcane molasses. Values represent means of three replicates ± standard error (vertical bars).

Table 1
Fermentation products after 72 h from sugarcane molasses by *Bacillus thuringiensis* and *Bacillus subtilis.* Values represent means of three replicates ± standard error.

Properties	*Bacillus thuringiensis* (g/L)				*Bacillus subtilis* (g/L)			
	sucrose	molasses			sucrose	molasses		
	15	40	60	80	15	40	60	80
Initial sugar (g/L)	15 ± 0.66	26.35 ± 0.20	49.02 ± 0.21	60.19 ± 3.52	15 ± 0.83	26.9 ± 0.34	49.7 ± 0.75	60.13 ± 1.13
Residual sugar (g/L)	2.12 ± 0.10	17.28 ± 0.62	34.64 ± 0.66	42.05 ± 1.17	5.88 ± 0.22	15.17 ± 1.72	35.1 ± 0.52	44.43 ± 1.43
Consumed sugar (g/L)	13.05 ± 0.54	9.07 ± 0.64	14.38 ± 0.27	18.14 ± 0.83	9.1 ± 1.15	11.73 ± 0.60	14.6 ± 1.58	15.7 ± 1.01
Total hydrogen (mL/L)	1400 ± 17.3	2000 ± 57.8	2450 ± 23.1	2320 ± 46.2	1200 ± 34.6	1830 ± 40.4	2300 ± 57.8	2150 ± 28.9
Hydrogen rate (mL/h)	19.44 ± 1.17	27.77 ± 1.19	34.02 ± 0.58	32.22 ± 2.7	16.66 ± 1.70	25.41 ± 2.13	31.94 ± 1.82	29.86 ± 1.06
H_2 mol/mol sucrose	1.16 ± 0.02	1.77 ± 0.04	1.37 ± 0.16	1.03 ± 0.04	1.06 ± 0.04	1.25 ± 0.04	1.27 ± 0.04	1.1 ± 0.04
Ethanol (g/L)	0.52 ± 0.01	0.52 ± 0.04	1.55 ± 0.03	0.41 ± 0.008	0.46 ± 0.02	0.5 ± 0.04	1.03 ± 0.05	0.187 ± 0.03
Acetic acid (g/L)	0.07 ± 0.002	0.45 ± 0.02	1.1 ± 0.03	0.36 ± 0.02	0.06 ± 0.01	0.34 ± 0.03	0.55 ± 0.04	0.29 ± 0.02
Lactic acid (g/L)	0.02 ± 0.001	0.05 ± 0.004	0.07 ± 0.003	0.014 ± 0.001	0.01 ± 0.001	0.25 ± 0.008	0.05 ± 0.002	0.045 ± 0.003
Butyric acid (g/L)	4.23 ± 0.38	4.89 ± 0.35	10.39 ± 0.17	5.9 ± 0.046	4.07 ± 0.04	5.6 ± 0.17	5.9 ± 0.11	5.2 ± 0.12

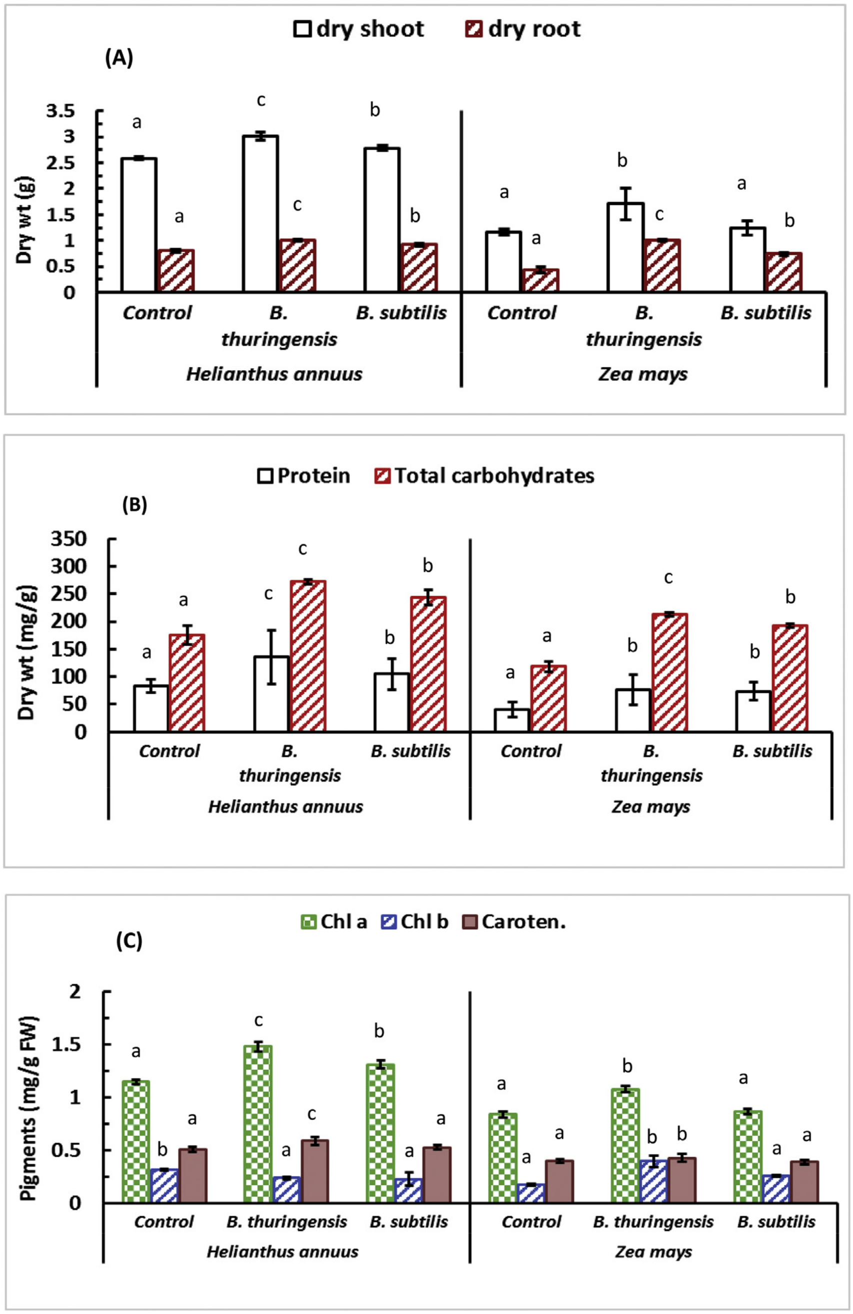

Fig. 6. **(A)** Dry weight, **(B)** Total carbohydrates and protein content, **(C)** Chlorophyll content of *Helianthus annuus* (sunflower) and *Zea mays* (Corn) plants. Values represent means of three replicates ± standard error (vertical bars). Means with the same letter are not significantly different among bacterial isolates at the 0.05 level using Duncan's multiple range tests.

3.7. Effect of N_2-fixing spent biomass of Bacillus thuringensis and Bacillus subtilis on the growth of two crop plants

Inoculation of sunflower and corn plants with diazotrophic spent bacterial biomass of *B. thuringensis* and *B. subtilis* lead to a significant increase in dry-matter of shoot or root accumulation over controls in all experiments (Fig. 6A). Sugar contents of plants either inoculated with *B. thuringensis* or *B. subtilis* were higher than uninoculated plants (Fig. 6B). The obtained results indicated that the total protein and carbohydrates content of two tested plants inoculated with *B. thurengensis* and *B. subtilis* were greater than those with control plants. Also, growth analysis indicated that the difference in growth rates between the two bacterial treatments was attributable to the amount of carbohydrates. Also, statistical analysis revealed that chlorophyll *a*, chlorophyll *b* and carotenoids content of the two tested plants inoculated with *B. thuringensis* and *B. subtilis* were higher than uninoculated plants (Fig. 6C).

The results of the current study matching with data obtained by de-Bashan et al. (2010) who reported that the growth (dry shoot and root) of *Atriplex lentiformis* inoculated with *Bacillus pumilus* ES4 was significantly enhanced than uninoculated crop plants. The significant increase in dry weight, total carbohydrates and pigments content of plants inoculated with tested spent bacterial biomass could be attributed to nitrogenase activity of these organisms (Fig. 5B). Utilization of N_2-fixing bacterial spent biomass as biofertilizer plays an indispensable role in maintaining soil fertility, upgrading plant growth, and development of a sustainable agricultural system for the production of agrochemicals-free and safe food. Sufficient inoculation of seeds with diazotrophic *Bacillus* spent biomass could provide a beneficial role in creating a proper environment for plant growth and enhance the availability of nutritionally important elements such as N, P and K as well as inhibiting pathogen growth (Abd-Alla et al., 1994). *Bacillus* spp. solubilize the complex form of essential nutrients such as phosphorus to a simple available form that is utilized by plant roots (Kang et al., 2015; Kuan et al., 2016). Nitrogen is an important component of proteins, nucleic acids and other organic compounds in plants and the available form of N_2 in soil is limited, which slows plant growth in natural habitats (Ohyama, 2010; Leghari et al., 2016). Biological N_2-fixation is one of the most important bacterial processes occur in the plant-soil environment. This process contributes to the availability of nitrogen in agricultural crops (Chanway et al., 2014; Aquilanti et al., 2004). The enhancement of growth and yield of inoculated crop plants by *Bacillus* spp. is due to the presence of the *nifH* gene in these bacteria which is responsible for atmospheric N_2-fixation and delivers it to the plants (Ding et al., 2005). Many investigations have shown that inoculation with *Bacillus* species significantly enhanced plant growth and can be used as a plant growth promoter for several crop plants (Kumar et al., 2012; Batistaa et al., 2018). *Bacillus* species have capabilities to colonize a variety of the soil and plant roots environment. These bacteria are characteristic by their ability to form endospores and thereby to persist and survive under drastic conditions (Toyota, 2015). Survivability and resistance to harsh conditions are always the issues that many microbiologists have attempted for years, and those are now the topic in the context of ecological impacts (Haruta and Kanno, 2015). While the inoculation of beneficial microorganisms has received more attention to enhance the productivity of crop plants (Toyota and Watanabe, 2013), the low survivability of the introduced bacteria is the main obstacle in their applications. According to the persistence and withstand characters of spore-forming *Bacillus* species, their inoculants to enhance crop growth have a great advantage over non-spore forming ones. Suppression of plant disease, nitrogen fixation and production of phytohormones are mechanisms of crop growth promotion by *Bacillus* species. Such inoculants that can be used for different plant species are of great agricultural interest due to their wide range of applications. Spent bacterial biomass, as a nutrient-rich organic by-product, is generated in considerable amounts coexisting with these high-value fermentation products. After bacterial fermentation is completed, spent bacterial biomass is removed and instead of incineration or inactivation (Halter and Zahn, 2016; Mathias et al., 2014), it will be reused as N_2-fixing biofertilizer for enhancement of crop plants growth (Sullivan et al., 2017).

4. Conclusion

Among twenty-one *Bacillus* isolates, four namely *B. thuringensis, B. subtilis B. pumilus* and *B. licheniformis* were the most efficient N_2-fixing organisms. The discrepancies between the efficiency of hydrogen producing *Bacillus* species could be attributed to the *nifH* gene expression which is the main source of hydrogen generation. The current study suggested that sugarcane molasses is a suitable substrate for the production of liquid and gas biofuel by *B. thuringensis and B. subtilis.* The maximum biofuel production was achieved at 6% molasses. For enhancement the economic feasibility and minimizing the cost of biofuel production, the spent bacterial biomass of *B. thuringensis* and *B. subtilis* (waste fermentation), consequent to biofuel production, were efficiently reused as N_2-fixing biofertilizer for inoculation of crop plants (sunflower and corn). Spent bacterial biomass significantly enhanced the growth of two crop plants. The reuse of the waste fermentation culture of N_2-fixing *Bacillus* species as biofertilizers offers a sustainable alternative to the current method of disposal or inactivation with heat or chemical treatment. Application of such waste fermentation biomass of N_2-fixing *Bacillus* as the N_2 source of agricultural crop production may reduce the environmental and economic footprint of synthetic fertilizers. Decreasing the cost of biofuel production technology through reuse of spent bacterial biomass as N_2-fixing biofertilizer in the agriculture system might enhance the economic feasibility of biofuel production technology.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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