A novel insight into the fabrication of Polyhydroxyalkanoates from actinobacteria *Streptomyces toxytricini* D2: screening, optimization, and biopolymer characterization

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Abstract

The objective of this study was to find a biopolymer producing potential actinobacteria candidate from polluted soil. A biopolymer fabricating *Streptomyces toxytricini* D2 was isolated and characterized by 16S rRNA sequencing. The *S. toxytricini* D2 produced polymeric granules were categorized by FTIR, 1H NMR, and 13C NMR analyses and confirmed that the produced granules were Polyhydroxyalkanoates (PHAs). The *S. toxytricini* D2 has the competence to fabricate 86.56% of PHAs (23.64 g L^{-1} of PHAs from 27.31 g L^{-1} of biomass) under the optimized growth conditions: 8% of tapioca molasses, 4% of $(NH_4)_2SO_4$, 8% of inoculum, pH 6.5, 30 ºC and 72 h of incubation. These results concluded that this is the novel report about the *S. toxytricini* D2 as could be used as an efficient candidate for the mass production of PHAs polymer under optimized conditions with low-cost carbon source and in a short duration of the production process.

1. Introduction

The wide applications such as building materials, equipment's, packaging, etc. nature of plastic, the requirements are raising every day in the entire word (Lai et al., 2020; Mohapatra et al., 2017). The continuous usage of non-renewable petroleum-based plastics would be exhausted very soon due to excess utilization and less recycle management technology (Dai et al., 2020; Pérez et al., 2020). Besides that, the frequent usages and unscientific disposal of these traditional polymers creating severe soil and water pollution (Alshehrei, 2019). Furthermore, while during accidental burning, it releasing toxic greenhouse gases into the environment and cause severe air pollution (Mohapatra et al., 2017). The abandoned used plastic materials persist in the environment for several years due to its less or non-degradability in nature (Huang et al., 2020; Xiong et al., 2020). However, the instant complete avoidance of these traditional plastics are not possible without finding an eco-friendly alternate material for traditional plastic (He et al., 2018). Fortunately, nature has to provide an excellent renewable sources through microbes and plants (Pérez et al., 2020). Among these, the microbes, especially the bacteria, is one of a preferable source for eco-friendly biodegradable plastic production (Girão et al., 2019; Zhang et al., 2020). The bacterial based biopolymer producing bacteria isolation and biopolymer production receiving more attention among researchers due to its multiple application as similar to traditional plastics and with fast biodegradable in nature (Banu et al., 2019). The significant variation among conventional polymers with biopolymers is possibilities of microbial degradation after utilization of them. Since the most percentage of biopolymers are made up of carbon and nitrogen, which could act as a nutrient for microbial growth and leads to reduce as a carbon dioxide and water through microbial enzyme activity (Zikmanis et al., 2020). Among various bacterial based polymers, the polyhydroxyalkanoates (PHAs) is a most preferable polymer as it could be synthesized by several numbers of bacteria as an energy backup nutrient components and protect the bacterial cell from adverse environmental stress conditions and limited quantity of essential elements such as nitrogen and phosphorous (Koutník et al., 2020). Numerous bacterial species such as *Bacillus* sp., *Alcaligenes eutrophus*, *A. latus*, *Protomonas extorquens*, *P. oleovorans*, *Azotobacter vinelandii, Escherichia coli*, etc.

had been reported as suitable candidates for the fabrication of biopolymer (PHA) (Kucera et al., 2018; Pérez et al., 2020). The poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-3-hydroxyvalerate (PHBV) are a major part of PHA (Banu et al., 2019). Most of the bacterial species accumulating these PHA molecules in the cell cytoplasm (Shanmugam & Abirami, 2019). These mobile, amorphous, and liquid granules present in the cytoplasm region of actinobacteria and might are support the growth of bacteria under environmental stress conditions biopolymers allowing microbial survival under stress conditions (Girão et al., 2019). However, the successful replacement of traditional oil-based polymers by biopolymer is limited due to the cost of mass production (Zikmanis et al., 2020). Hence to sort out these issues, finding a cheap raw material (carbon and nitrogen sources) utilizing and biopolymer producing bacterial candidate is timely needed. Therefore in this study, apart from common bacterial species, the actinobacterial species (*S. toxytricini* D2) isolated from industrial effluents polluted site has been investigated to assess their PHA producing competence and it could be foremost report. Very few number of actinobacterial species such as *Kineosphaera limosa*, *Rhodococcus* sp., *Nocardia* sp., etc. are found as fine PHA accumulating actinobacteria (Singh Saharan et al., 2014). The PHAs produced intracellularly and stored as carbon and energy reservoir by several bacteria. The PHA accumulated in the actinobacteria is located in the cell cytoplasm, mobile, amorphous, and this liquid granules are allowing the actinobacteria survival under abiotic stress conditions including nutritional factors and the presence of excess carbon source (Sathiyanarayanan et al., 2013). Based on the aforementioned perspectives, this research was designed to isolate the most active PHA producing actinobacteria species from polluted soil and characterized the PHA and optimized the growth parameters.

2. Materials And Methods

2.1 Collection and processing of soil sample

The Hosur SIPCOT region has been chosen for this study, since the bacteria which exists in the contaminated soil, for the successful survival they should possess the competence to produce the biopolymeres like molecules (Zikmanis et al., 2020). With this presumption, about 10 g of polluted soil from four different SIPCOT region was collected from 5–10 cm depth individually in ethanol sterilized glass container. The sample collected containers were immediately transferred to the laboratory and performed the studies continuously.

2.2 Enumeration of Actinobacteria culture

The enumeration of indigenous actinobacteria from polluted soil sample was achieved by standard serial dilution process. About 0.1 ml of 10^{-5} dilution from each (four) samples were inoculated on actinobacteria isolating selective media (YIM 7 HV medium) by spread plate method (Girão et al., 2019). The YIM 7 HV medium (pH 7.2) contain 0.5 g of Disodium hydrogen phosphate, 1.0 g of Humic acid, 1.7 g of potassium chloride, 0.05 g of Magnesium sulfate, 0.01 g of Ferrous sulfate heptahydrate, 1 g of Calcium dichloride 0.5 mg of each B-vitamins, 20 g of agar for 1000 ml distilled water. The inoculated plates were incubated at 30°C for 48 h with proper labelling.

2.3 Screening of biopolymer producing actinobacteria

2.3.1 Primary screening

In the initial screening process total of 17 active cultivable actinobacteria isolates were isolated from YIM 7 HV medium. The biopolymer producing potential of these isolates was analysed (Rendón-Villalobos et al., 2016). Briefly, the pure form of these 17 well grown colonies were individually treated (poured over the grown colonies) with 2.5 ml of 0.05% of Sudan Black B stain and kept undisturbed for 30 min at room temperature and then rinsed with 60% ethyl alcohol. The Sudan Black B stained plates were incubated at room temperature for 35 min and observed the formation as black greenish-blue color, deliberated as positive for PHAs biopolymer.

2.3.2 Secondary screening

The PHA primary screening results revealed that among 17 predominant isolates, the isolate H09 only indicated as a positive for PHA producer. The PHA producing potential of this isolate was identified by exposed to the PHA selective media (0.25g of potassium dideuterium phosphate, 2.5 g of di-sodium hydrogen phosphate dehydrate, 10 g of mannitol, 2 g of sodium chloride, 0.1 g of magnesium sulphate, 10 g of sodium pyruvate, 1 g of peptone, 0.12 g of bromothymol blue, and 2 g of agar) test. A loop full of test isolate (H09) was inoculated on this PHA selective media containing plate and incubated at 30°C for 48 h. The development of deep bluish-black colonies was identified as PHA positive isolate (Krishnan et al., 2017).

2.3.3 Tertiary screening by morphological analysis

The Transmission Electron Microscope (TEM) (TEM- Talos F200i Thermo Fisher, Mumbai, India) was used to finalize the PHA producing potential of test isolate H09 by following the standard protocol (Nwinyi & Owolabi, 2019). Briefly, about 2.5 mL of test (H09) isolate (1×10^2 cells mL^{-1}) at exponential phase was fixed with glutaraldehyde (4%), subsequently with osmium tetroxide (1%) and desiccated with 80–100% of acetone by consecutive treatment. The dried and fixed cells were submerged in epoxy resin and kept at sample boat, and the resin was polymerized at 60°C for 24 h. Sub sequentially, the resin-coated fixed cells were stained with uranyl acetate (22 min) and lead citrate (5 min) and observed under TEM.

2.4. Molecular characterization of H09

The results attained from the screening studies stated that the only one isolate (H09) out 17 has the potential to produce PHA. The basic cultural and morphological characteristics were studied, and the test culture was suspected as might belongs to actinobacteria group. Hence, the 16s rRNA sequencing was achieved to recognize the genus and species of test isolate (H09). Briefly, the standard RNA extraction kit from SRL Chemicals, Pvt. Ltd, Mumbai, India has been used to extract the RNA and amplified with standard universal primer (8F (5'-AGAGTTTGATCCTGGCTCAG-3'). The PCR amplification conditions were optimized as: denaturation: 95 ºC (2 min), annealing: 55 ºC (30s), and extension was at 72 ºC (10 min)

with 30 cycles. The amplified PCR component was decontaminated by the PCR product purification kit (SRL Chemicals, Pvt. Ltd, Mumbai, India) and sequencing system (518F/800R) was used for sequencing the purified PCR product (Nwinyi & Owolabi, 2019). The aligned sequences were identified at the National Center for Biotechnology Knowledge (https:/blast.ncbi.nlm.nih.gov/ Blast) in regions of local similarity with Genbank database by nucleotide BLAST. Based on similarly scores with registered species, the isolated species were described by Neighbour-joining method max sequence difference of 0.75 distance using BLAST. Furthermore, the sequence was registered at NCBI and received accession numbers as MT228958.1 (Kumar et al., 2020).

The MFE secondary structures of *Streptomyces toxytricini* D2 was predicted using the available online RNAWebSuite/RNAfold bioinformatics tools. The following link was used http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (Bhattacharjee et al., 2012). To assess the possibilities of genetic modification in this attained *S. toxytricini* D2 was studied by bioinformatics tool. The restriction sites in 16S rRNA of *S. toxytricini* D2 was analysed using NEB cutter programme version 2.0. The following links were used http://nc2.neb.com/NEBcutter2/cutshow.php?name=6e610b1b-MT228957.1 (Hajiaghayi et al., 2012).

2.5. Extraction of PHA from *Streptomyces toxytricini* D2

The PHA fabricating test isolate was found as *Streptomyces toxytricini* D2 by 16S rRNA sequencing. For the characterization of PHA molecule synthesised by *S. toxytricini* D2 was extracted (Alarfaj et al., 2015). Concisely, the biomass of *S. toxytricini* D2 (earlier or middle stationary phase) from YIM 7 HV broth medium was extracted by spun at 10000 rpm for 8 min. Then pellet was treated 1 min with acetone and ethanol (1:1 ratio) to break the cell wall and to extract the PHAsgranules from *S. toxytricini* D2 and subsequently centrifuged at 10000 for 3 min and discarded the supernatant. About 1 mL of 4% NaOCl was added to dissolve the pellet and kept undisturbed for 30 min at room temperature and centrifuged at 10000 rpm for 5 min. around 2:1 ratio of acetone and ethanol was used to wash the pellet. Then the polymers containing pellet was dissolved with sufficient quantity of chloroform and filtered by filter paper (Whatman No. 1). The polymers containing filtrate was treated with 5 mL of concentrated sulfuric acid to reduce the polymeric granules into crotonic acid. The optical absorbance value of this sample was read at 240 nm using UV-Vis spectrophotometer (UV/Vis/NIR-LAMBDA 1050+, PerkinElmer, USA), and with various concentration of commercial PHA (SRL, Chemicals Pvt. Ltd., Mumbai, India) molecule was used as a reference.

2.6. Characterization of PHA extracted from *S. toxytricini* D2

2.6.1. Fourier Transform Infrared Spectroscopy study

The polymeric granules produced and extracted from *S. toxytricini* D2 was subjected to FTIR analysis (Alarfaj et al., 2015). Briefly, a portion of crude PHA (1 mg) extracted from procedure above was dissolved with chloroform (5 mL) and mixed gently for few minutes. About few drops of the sample was smeared on FTIR KBr disk (PerkinElmer, spectrum 3™ Tri-Range FTIR). Then the spectra of solvent evaporated PHA

extract and were read at decent resolution (4 cm^{-1}) with vacuum pressure of 400–4000 cm^{-1} by using IR double beam spectrophotometer.

2.6.2. ^{1}H NMR analysis

The monomers (1H) contents of PHA molecules produced by *S. toxytricini* D2 was characterized using ^{1}H-NMR analysed (Alarfaj et al., 2015). About 10 mg of crude PHA granules were dissolved with 1 mL of chloroform, and Tetramethyl saline was utilized as an internal reference. The spectra of liquefied PHA molecules were recorded in DMSO on Bruker ACF300 spectrophotometer at 300.53 MHz frequency with the resolution of > 0.1 deg and > 0.1 Hz respectively.

2.6.3. ^{13}C NMR analysis

The carbon skeleton of 20 mg of concentrated PHA (CDCl3 dissolved) molecules extracted from *S. toxytricini* D2 was recorded at 30°C by ^{13}C NMR (Baran et al., 2018) using a 500 MHz NMR spectrometer (Bruker ACF300 spectrophotometer: has ^{1}H and ^{13}C dual probe) with the MAS frequency of 170 MHz and 10 kHz correspondingly.

2.7. Growth parameters optimization for PHA synthesise

To assess the PHA secreting extent potential of *S. toxytricini* D2, the basic growth parameters and find low cost nutritional factors were studied as one-factor-at-a-time mode (Narayanan et al., 2020). The parameters such as various concentrations (2, 4, 6, 8, and 10%) of different carbon (w / v) sources such as pre-treated tapioca molasses, sugarcane molasses, and pulverized rice bran. The different concentrations (1, 2, 3, 4, and 5%) of various nitrogen sources such as $(NH_4)_2SO_4$, NH_4NO_3, and yeast extract were added in minimal medium (contain: 0. 5 g of $MgSO_4$ $7H_2O$, 0.5 g of NaCl, 1 g L^{-1} of K_2HPO_4). Various percentages (2, 6, 8, 10, and 12%) of inoculum of *S. toxytricini* D2 (1 x 10^6 CFU mL) were studied. The various degrees of temperature (25, 30, 35, and 40 ºC), different pH (6.5, 7.5, 8.5, and 9.5) and various incubation time (24, 48, 72, and 96 h) were evaluated. The each parameters were achieved with triplicates to attain results accuracy and reproducibility. The bacteria inoculated with parameters mentioned above containing media were incubated on a shaker incubator with 150 rpm for up to 96 h. The amount of PHA produced (extracted by previous method), and growth of *S. toxytricini* D2 from each parameter was studied at 240 nm and 600 nm respectively by UV-Vis spectrophotometer on day interval basis (Getachew & Woldesenbet, 2016).

2.8. Growth kinetics and production of PHA under optimized conditions

The optimized growth conditions for the maximum PHA production by *S. toxytricini* D2 were 8% of tapioca molasses, and 4% of NH_4NO_3 were served as suitable carbon and nitrogen sources respectively. Furthermore, 8% of inoculum, 30 ºC, pH 6.5, and 72 h of incubation were optimized as suitable growth conditions for PHA fabrication by *S. toxytricini* D2. The maximum PHA producing potential of *S. toxytricini* D2 was studied by inoculating 6% of *S. toxytricini* D2 (1 x 10^6 CFU mL) on minimal broth

medium compiled with optimized parameters (8% of tapioca molasses, 4% of NH_4NO_3, 30 ºC, pH 6.5) and incubated in a shaker incubator for 72 h with 120 rpm. The growth kinetics and PHA production were recorded by method as mentioned earlier (Krishnan et al., 2017).

2.9. Statistical analysis

All the experiments were performed in triplicate mode to achieve accuracy and reproducible results. Furthermore, the mean, standard error, and one- way ANOVA were performed using SPSS. Software version 13.

3. Results And Discussion

3.1. Brief profile of soil sample collected site

The soil samples collected sites have been polluted by various industrial activities, since it is an industrial area. Hence the bacteria that survive in this abiotic stress environment as a lifesaving mechanisms the bacteria could secrete different kinds' high molecular weight molecules such as polysaccharides, polyamides, polyesters, and polyphosphates. These polymeric components can act as protective capsular layers for bacteria that survive under abiotic stress conditions (Moradali & Rehm, 2020). The synthesis and mobilization of PHA in bacteria have been directly related to environmental stress and fit for survival under abiotic stress (Niether et al., 2020). These metabolically adapted bacterial strains could produce fine quality and quantity of biopolymers.

3.2. Enumeration of biopolymer producing predominant isolate

About 17 bacterial isolates have been screened from a polluted soil sample collected from an industrial site. Different morphological characteristics bacteria were observed, nevertheless in the initial Sudan Black B stain screening study stated that only one bacterial strain was showed dark blueish black color colonies and this was deliberated as positive for PHA producer (Mostafa et al., 2020). The Sudan black B can stain the lipid or lipid-associated polysaccharide material (polymeric) and produced deep blue-black color. On secondary screening in PHA selective media, deep bluish-black colonies of H09 was observed in the well-grown plate.

As a final confirmation, the TEM analysis was achieved to identify the PHA producing potential of isolate H09. The TEM images of test isolate H09 were showed that the presence of polymeric granules (PHA) located at the close region of the cytoplasmic membrane (Fig. 1). Most of the produced PHA granules were uniformly oval and elongated in shape with clear boundary line of each granule were seen in the cytoplasmic region of test isolate H09. Similarly, halophilic archaea isolated from polluted soil produces spherical, ovoid, and elongated shaped granules (Danis et al., 2015). This multi-shaped granules might cause little complications in the purification process. Fortunately, the granules produced by test isolate

H09 showed identical in shape that could be considered as more advantage of this test isolate for mass production with fine quality polymeric substances.

3.3. Genomic identification of test isolate

The PHA was producing test isolate H09 was genotypically characterized using 16S rRNA analysis. The amplification characteristics of the 16S rRNA gene of isolate H09 were carried out by PCR and sequencing systems. The 1400 bp 16S rRNA genes sequence of isolate H09 was a blast and registered in GenBank http://www.ncbi.nlm.nih.gov/genbank and acquired the accession number from NCBI as *Streptomyces toxytricini* D2 (MT228958.1). The partial sequence of the *S. toxytricini* D2 (MT228958.1) was compared with sequences of already existing genera of actinomycetes from NCBI database to determine the phylogenetic relatedness clustered together as one clade segments corresponding to an evolutionary distance of 0.002 and 0.009 as shown with bars by using Neighbour-joining method (Fig. 2). It was revealed that the sequence of the isolate *S. toxytricini* D2 found 99% similarity with the existing species of *S. toxytricini* strain HBUM174624 (Fig. 2). The results were directly compared with the previous findings (Charousová et al., 2018; Salgaonkar & Bragança, 2017). They isolated commercially valuable *Streptomyces* sp. from polluted soil and characterized through 16S rRNA sequencing and compared by phylogenetic analysis. The Minimum Free Energy (MFE) secondary structure of the 16S rRNA gene of *S. toxytricini* D2 (MT228958.1) showed 27 stems in their MFE structure (Fig. 3). However, *S. toxytricini* D2 has GC-59% and AT-41% (Fig. 3). These observations also agree with the results reported by researchers on *Streptomyces* sp. isolated from soil sample (Heinsch et al., 2019). The *S. toxytricini* D2 is a mesophilic soil bacteria and previously reported as a suitable candidate for commercially valuable enzyme production (Kumar et al., 2020). The genetic modification in *S. toxytricini* D2 is possible due to appropriate restriction sites and its fine GC content could balance the stability to broad growth conditions (Bhagowati et al., 2015). Thus, this genomic trait of *S. toxytricini* D2 could support the biopolymer commercial production.

3.4. Categorisation of PHA produced by S. toxytricini D2

The polymeric extracted from *S. toxytricini* D2 was successfully removed and confirmed as PHA by compared with reference PHA (commercially available) molecule by UV-Vis spectrophotometer with absorbance in the range of 200 to 360 nm (Sathiyanarayanan et al., 2013). The highest absorbance peak for PHA extract was recorded at 240 nm (OD value: 2.46) and it was almost identical to commercial PHA (OD value: 2.40) and it confirmed that the extracted polymer was PHA molecule (Fig. 4). Similarly, the polymeric granules are also removed from the *Bacillus subtilis* NCDC0671 and confirmed that molecule as PHA through UV-Vis spectrophotometer analysis (Umesh et al., 2018).

3.4.1. FT-IR

The figure. 5 displays the FTIR spectrum of the Polyhydroxyalkanoates as extracted from *S. toxytricini* D2. The absorption band appeared at around 3500 cm^{-1} associated with the free-hydroxyl group's stretching frequency in the polymer chain (Alarfaj et al., 2015). The multiple peaks were observed

between 2800 and 3100 cm^{-1} corresponding to the symmetric and asymmetric stretching vibrations of -CH_3 and -CH_2-CH_3 alkane groups. Furthermore, the low intensity of –-CH_3 peak attributes the crystallisation process's conformational disorder (Biradar et al., 2018). Interestingly, the absorption band of carbonyl functional group was observed as the doublet of ketone group (C = O) at nearly 1742 cm^{-1} and amide group (N-C = O) at 1660 $cm^{-1,}$ corroborates the stretching vibrations of carbonyl ester and intracellular amide of microbes respectively (López-Cuellar et al., 2011; Mostafa et al., 2020). The terminal methyl group (-CH_3) was confirmed from the intense peak observed at 1379 $cm^{-1,}$ and the cluster of absorption peaks appeared at below 1200 cm^{-1} might be associated with the stretching frequency of –C-O-C-, -C-O and –C-C- functional groups (Getachew & Woldesenbet, 2016). The FTIR spectrum's designated absorption peaks (Fig. 5) reveals that the formation of PHA polymer in amorphous phase with a trace amount of impurities from the starting materials and well correlated with the reported literature (Bhatt et al., 2008; López-Cuellar et al., 2011).

3.4.2. ^{1}H NMR and ^{13}C NMR analysis

The structural specifics of *S. toxytricini* D2 produced PHAs were studied by ^{1}H NMR and ^{13}C NMR analyses. The figure. 6 shows the ^{1}H NMR spectrum of the PHAs produced by *S. toxytricini*. The resonance signal observed at 5.25 ppm was due to methylene protons adjacent to carboxyl groups of HB (Linton et al., 2012). Whereas the multiplet resonance of protons of methylene and methane of α-carbon were observed at 2.5 ppm. Peaks at 1.45–1.52 ppm were observed concerning the methylene protons adjacent to the β-carbon of the saturated side chain (Bhuwal et al., 2013; Singh et al., 2011). The predominant peak observed at 1.23 ppm is ascertained to the presence of methyl protons of the side chain.

The figure. 7 shows the ^{13}C NMR spectrum of PHAs produced by *S. toxytricini* D2. The carbon resonance peak observed at 22.72 ppm was due to the presence of methyl carbon. The peaks observed from 43.09 to 45.2 ppm were due to the saturated side chain's methylene carbon. Further peaks were observed at 138.5 ppm for methylene carbon attached to a carboxylic acid group. At 173.10 ppm carboxyl carbon peak was observed (Sabarinathan et al., 2018; Yalpani et al., 1991). Thus the results of ^{1}H and ^{13}C NMR spectra indicated that the intracellular molecules produced by *S. toxytricini* D2 were mostly identical with the PHAs.

3.5. The growth parameters optimization for PHA production by *S. toxytricini* D2

The favorable growth conditions are the most significant factor in attaining the expression of the maximum potential of all organisms (Charousová et al., 2018). Similarly, the bacteria also required the most suitable growth conditions for more human and environmental welfare products such as PHA (Krishnan et al., 2017). In this study, the optimal growth requirements such as the various concentration of various low-cost carbons sources (tapioca molasses, sugarcane molasses, and pulverized rice bran), nitrogen sources ($(NH_4)_2SO_4$, NH_4NO_3, and yeast extract), different percentage of inoculum, temperature,

pH, and different incubation time (one factor at one time) and triplicates were performed for each parameter analysis.

3.5.1. Suitable carbon and nitrogen sources

For the effective and quality microbial production process, generally, the microbes require carbon and nitrogen sources as most significant factor and it determines the quantity and quality of microbial products such as biopolymers (PHA) (Krishnan et al., 2017). The carbon and nitrogen sources are the most significant microbial product quality and quantity determining factors (Mohapatra et al., 2017). The *S. toxytricini* D2 effectively utilized and produced PHA (86.65%) at 8% concentration of tapioca molasses as 17.34 g L^{-1} of PHA from 20.01 g L^{-1} of cell biomass. It was statistically significant ($p > 0.03$) to other concentration and carbon source. The pulverized rice bran, and sugarcane molasses were acted as a fine carbon source for *S. toxytricini* D2 and produced 80.18% and 69.81% of PHA at 10% concentration of both carbon sources, respectively (Fig. 8a to 8c). At 10% concentration, these acquired value were statistically significant at $p > 0.05$ than other concentrations (2 to 8%) of pulverized rice bran and sugarcane molasses. About 4% of $(NH_4)_2SO_4$ was served as a most suitable nitrogen source for PHA (66.02%) production in *S. toxytricini* D2 as produced 8.2 g L^{-1} of PHA from 12.42 g L^{-1} of cell biomass, and it was statistically significant ($p > 0.03$) to other concentration and nitrogen source (Fig. 8d to 8f). The *S. toxytricini* D2 produced 51.47 and 61.85% of PHA from 4% of NH_4NO_3 and yeast extract correspondingly. The statistical significance of these values among the remaining concentrations were $p > 0.05$. These results indicate that even though the carbon and nitrogen sources are essential factor, when their concentrations increase, the bacteria could not utilize them all. Furthermore, the presence of excess concentration might reduce cell viability and activity (Pérez et al., 2020). Thus the sufficient quantity of suitable nitrogen and carbon sources could enhance bacterial metabolic activity and growth. It leads to a reasonable quantity of bacterial products (PHA) with fine quality (Rendón-Villalobos et al., 2016). Besides that, the carbon and nitrogen-based substrate determine the type of polymeric component (PHAs and PHBs), since the quantity of carbon atoms exist in the biopolymers are typed into two as 3–5 carbon atoms based PHAs (Short Chain Length PHAs: SCL PHAs) and 6–14 carbon atoms based PHAs (medium-chain length: MCL PHAs), due to the activity of substrate specific PHA synthases that can recognize 3-hydroxyalkanoic acid with assured range of carbon length (Shanmugam & Abirami, 2019). The MCL PHAs possess more elasticity and low melting temperature with least degree of crystallinity in nature (Zikmanis et al., 2020). The SCL PHAs are termed as polyhydroxybutyrate. It has a high degree of crystallinity (> 50%) with thermoplastic in nature (high melting temperature: 180°C). Some other low-cost materials also previously reported such palm (Gabr, 2018; Khiyami et al., 2011) had been recorded as suitable carbon source for bacterial (*Bacillus* sp.) based biopolymer (PHA and PHB) production (58%). The maximum volume of PHAs could be synthesized under the limited dosage of nitrogen contents (Patel et al., 2017). It was correlated with this study's findings since more quantity of PHAs produced at 4% concentration of $(NH_4)_2SO_4$ than 5%. The limited nitrogen sources with readymade form could improve the biopolymer yield (Kourmentza et al., 2017).

3.5.2. Percentage of inoculum

The percentage of inoculum applied on the microbiological fermentation process received more attention as it determine the volume of yield and time duration of production process (Charousová et al., 2018). In this study, about 8% of inoculum of *S. toxytricini* D2 produced a reasonable quantity (66.7%) of PHAs (12.34 g L^{-1} of PHAs from 18.5 g L^{-1} of cell biomass) in a short duration of incubation than other dosages (Fig. 8g). It was statistically significant ($p > 0.03$) to other concentration. It followed by 10% of inoculum produced 62.74% of PHAs (Fig. 8g). The high dosage of inoculum might minimize the lag phase of the bacterium and enhance it to reach the log phase. There the PHAs production gets initiated; thus the short duration of the cell biomass could produce more volume of PHAs (Bhatt et al., 2008). Nevertheless, less PHAs yield was noted in 10% inoculum since at the inoculum concentration the cell might utilize more volume of nutrients during the lag phase itself (batch typed fermentation). At the same time, it reached to the log phase it faces nutrient depletion leads to less production (López-Cuellar et al., 2011). Similarly, the low PHAs yield was recorded at a low concentration of inoculum; it could be lag phase might take some more time to reach the log phase with average cell biomass and it leads to low PHAs yield (Mostafa et al., 2020). The dosage of inoculum for each bacteria for the production (fermentation) process should differ (Alshehrei, 2019). Accordingly, about 12.5% of inoculum of *Bacillus* sp. was found as an optimized dosage for biopolymer (PHAs and PHBs) production (Getachew & Woldesenbet, 2016).

3.5.3. The suitable temperature

Among the various physical parameters, the temperature plays a most significant role in the active growth of microbes (Girão et al., 2019). It determines the yield of biomass and microbial products since, under the optimized temperature, only the bacteria can continue their metabolic process (Johnson et al., 2010). In this study, the optimal temperature for significant growth and PHAs producing competence of *S. toxytricini* D2 was found as 30°C. The PHAs yield was observed as 72.95% (10.36 g L^{-1} of PHAs from 14.2 g L^{-1} of cell biomass) at 30°C and followed by 35°C (Fig. 8h), and it was statistically significant ($p > 0.03$) to other temperature. Low PHAs and biomass yield were recorded at 25°C (37.06%) and 40°C (62.74%). Several researchers have been reported that the optimal temperature for PHAs production through various bacterial species fell within the range of 25–35°C (De Grazia et al., 2017). The extreme and low temperatures might reduce the feast phase of bacteria along with swift nutrients uptake rates. Besides that, the O_2 transmission is more proficient at average temperatures since the higher volume of dissolved oxygen persists at average temperature (25–35°C). In mass production and economic aspects, the average temperature for the production process could minimize the cost of production (Chan et al., 2017). Similarly, species of *Nacardiopsis* and *Vibrio* have effectively produced biopolymers at 30 ºC (Mahitha & Madhuri, 2015). The optimal temperature might vary from species to species. According to this, the *Bacillus* sp. (Alshehrei, 2019), *E. aquimaris* (Mostafa et al., 2020), *S. thermophilus* (Kalaivani & Sukumaran, 2013), and produce a fine quantity of PHAs at 30 ºC, 35 ºC, and 50 ºC respectively. This temperature differentiation might be related to the temperature of bacteria isolated sites, since the enzymatic and metabolic activity could be significant (Pérez et al., 2020).

3.5.4. Optimal pH

The pH is another most essential factor that regulates the cell metabolic process. Since each enzymatic mechanisms in the cell has been directly related to the pH of the medium. More microbial products is obtainable under the optimal pH condition (Sasidharan et al., 2015). Accordingly, *S. toxytricini* D2 produced 67.42% (8.26 g L^{-1} of PHAs from 12.25 g L^{-1} of cell biomass) of PHAs at pH 6.5, and it was statistically significant ($p > 0.03$) to other pH (Fig. 8i). It might be the PHAs syntheses enzymes of *S. toxytricini* D2 effectively produce and accumulate the PHAs at slightly acidic pH; it chelates the further metabolic process and finally yielded a reasonable volume of PHAs. Similarly, *Ralstonia solanacearum* produces more biopolymer (PHBs and PHAS) acidic conditions (Macagnan et al., 2017). The optimal pH for bacteria might differed for each for PHA production. Similarly, *B. cereus* produce high yield of polymeric molecules at pH 7.5 (Macagnan et al., 2017), and for *Bacillus* sp (F15) pH 7 (Alshehrei, 2019). Moreover, some bacterial species might has the potential to grow under wide range of pH as acidic to alkaline, for that they developed particular adaptation strategy according to the pH and balancing the activity of enzymes such as ATP synthase, terminaloxidase (Bhatt et al., 2008), PHAs synthase (Baran et al., 2018) etc. These adaptation strategies permit the bacteria to balance the pH of cytoplasmic content and regulate optimal cell functioning.

3.5.5. Suitable incubation time

To attain the extent beneficial products from microbes, it need to be maintained for sufficient incubation time. In this study, the isolated actinobacteria *S. toxytricini* D2 produce the maximum yield of PHAs on 72 h of incubation. The PHAs yield was recorded as 57.58% (7.21 g L^{-1} of PHAs from 12.52 g L^{-1} of cell biomass), and it was statistically significant ($p > 0.03$) to other incubation time (Fig. 8j). The optimal incubation time for the growth of bacteria might differ from each other. According to this, the *Bacillus* sp. Ti3 produced 51.6 % of PHAs at 24 h of incubation time (Bhagowati et al., 2015), *Bacillus* sp. INT005 (35.30% in 48 h) (Tajima et al., 2003), *Bacillus cereus* SPV (38.0 % in 48h) (Valappil et al., 2008), *Bacillus mycoides* (50% in 72 h) (Soam et al., 2012), *Pseudomonas* sp, *Bacillus* sp, and *Rhizobium alti*, 40 h (Sathiyanarayanan et al., 2013). The optimized incubation time increasing 1.82 fold of biopolymer production by *Bacillus mycoides* DFC1 at 72 h of incubation (Narayanan & Ramana, 2012).

3.6 Production of PHA under optimized conditions

The growth kinetics and extent PHAs producing potential of *S. toxytricini* D2 were studied with optimized growth conditions such as 8% of tapioca molasses, 4% of $(NH_4)_2SO_4$, 8% of inoculum, pH 6.5, incubated at 30 ºC for 72 h of incubation period. Under these optimized conditions, the *S. toxytricini* D2 yielded 86.56% of PHAs as 23.64 g L^{-1} of PHAs from 27.31 g L^{-1} of cell biomass on 72 h of incubation (Fig. 9). The quantity of PHAs and cell biomass (growth kinetic) were similarly increasing from 24 h onwards and it was continued up to 72 h of incubation. Furthermore, the growth of *S. toxytricini* D2 was get decreased from 72 h on; it indicated that the lag phase of this actinobacteria might take around 24 h, and noticed absence or least amount of PHAs (Fig. 9). The figure. 9 revealed that on after 24 h of incubation the PHAs was gradually increasing along with cell growth and it declared that these actinobacteria reached the log phase at after 24 h of incubation and concurrently PHAs get produced on the same time onwards. The growth was reduced on 72 h of incubation and the PHAs production also halted on the same time of

incubation, it confirmed that the cell had reached lag stationary and decline phase. The attained yield of PHAs from *S. toxytricini* D2 was statistically significant with the cell biomass as $p < 0.003$.

Moreover, several numbers of bacterial species have been reported about the PHAs production under optimized conditions, the bacteria such as *Ralstonia eutropha* JMP 134 (50% of PHAs), *Bacillus cereus* FA11 (48.43%), *Rhodococcus aetherivorans* IAR1 (58.9%), *Haloferax mediterranei* (65%), *Halomonas boliviensis* LC1 (56%), *H. boliviensis* LC1 (88%), *Synechococcus* sp. MA19 (55%) etc. (Maskow & Babel, 2000; Ni et al., 2010; Singh Saharan et al., 2014). Very few reports are available in the *Streptomyces* sp. as biopolymer producers such as *S. griseorubiginosus*, *S. coelicolor*, *S. antibioticus, S. venezuelae, S. hygroscopicus* (Villano et al., 2010), *S. aureofaciens*, *S. griseus, S. parvus, S. albus*, etc. (Singh Saharan et al., 2014) all of this have reported as PHB producer. No previous reports are available about the production of PHAs from *S. toxytricini* D2. Thus this is the foremost study and reported about the PHAs producing potential of *S. toxytricini* D2 isolated from polluted soil.

4. Conclusions

The bacteria enumerated from polluted soil was identified as actinobacteria that have the potential to produce PHAs, and the exact genus and species of this isolate was identified as *S. toxytricini* D2 through 16S rRNA analysis. Successive screening studies confirmed the PHAs producing potential of this isolate, significantly the TEM analysis confirmed that the *S. toxytricini* D2 has the potential to produce PHAs. The FTIR, ^{1}H NMR, and ^{13}C NMR analyses were performed for structural confirmation of the polymers produced by *S. toxytricini* D2. The PHAs related signals and peaks were recorded in these technical, structural analyses (FTIR, ^{1}H NMR, and ^{13}C NMR). The suitable growth conditions for *S. toxytricini* D2 were optimized as 8% of tapioca molasses, 4% of $(NH_4)_2SO_4$, 8% of inoculum, pH 6.5, 30 ºC and 72 h of incubation. The *S. toxytricini* D2 produced 86.56% of PHAs (23.64 27.31 g L^{-1}: PHAs/cell biomass) on 72 h of incubation. The final results conclude that this is the major study on *S. toxytricini* D2 and reported that this actinobacteria has the excellent potential to produce PHAs molecules by consuming low-cost carbon source and in a short duration of incubation period. Thus, it might be a suitable candidate for making large quantity of PHAs molecules under optimized conditions. The assessment of commercially (PHAs) producing possibilities of this *S. toxytricini* D2 is under progress.

Declarations

Acknowledgment

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Figures

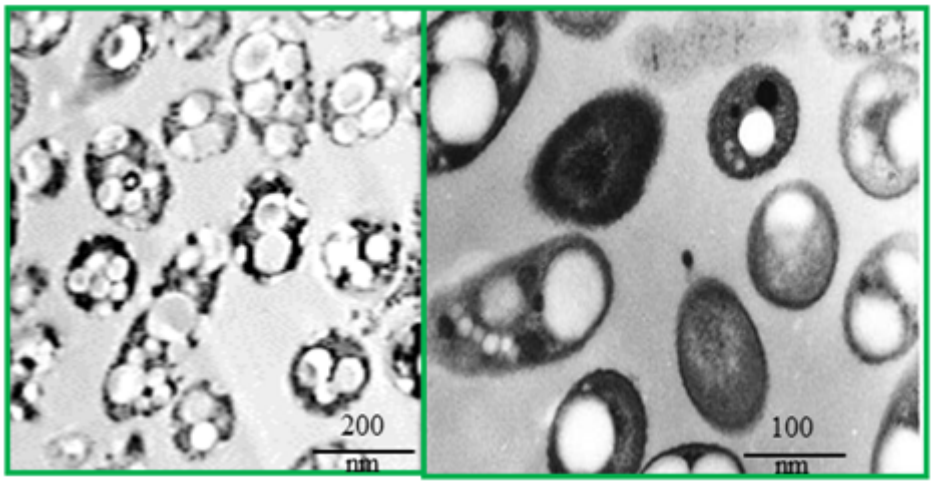

Figure 1

TEM analysis with various magnifications. Ovoid with elongated shape PHAs granules observed in test isolate H09

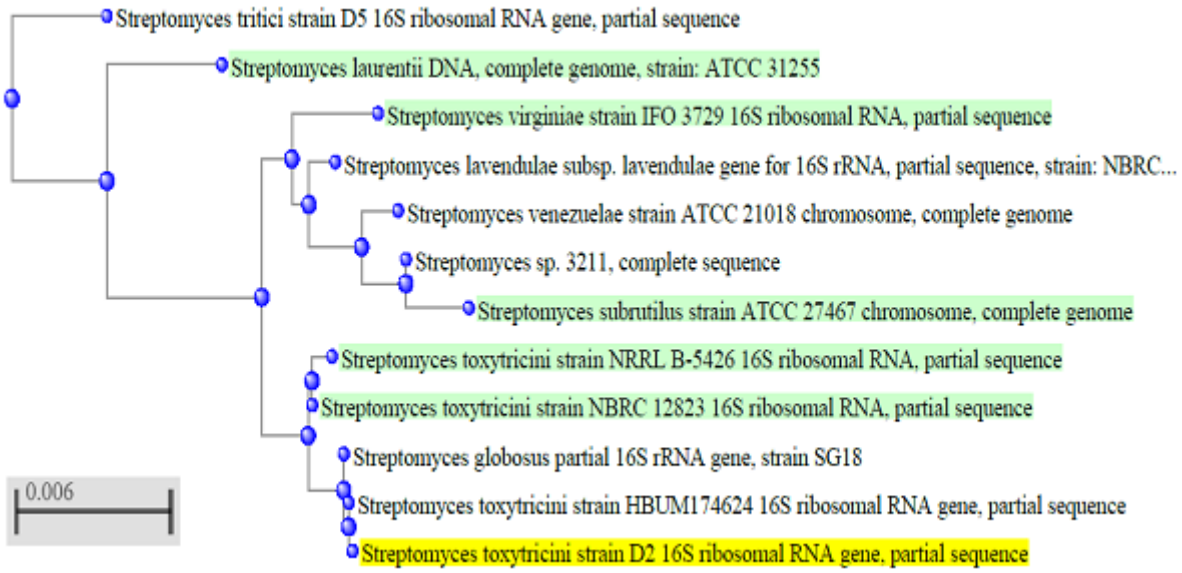

Figure 2

Phylogenetic analysis of 16S rRNA sequence of S. toxytricini D2

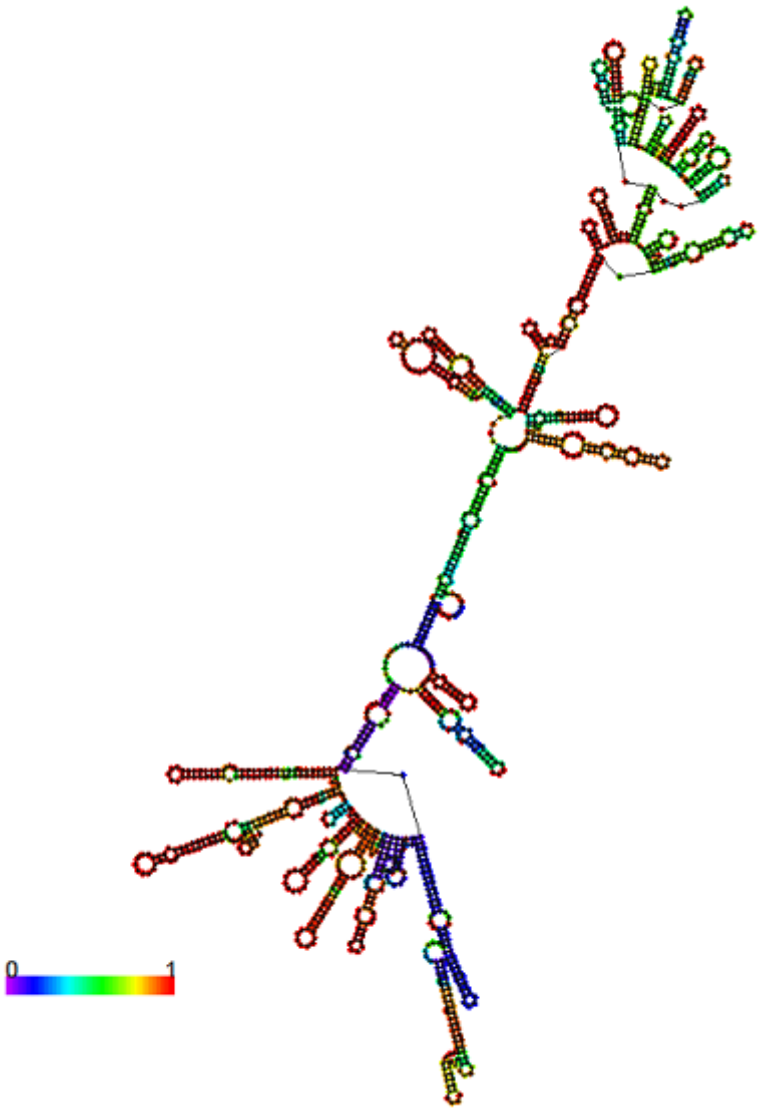

Figure 3

MFE secondary structure of S. toxytricini D2

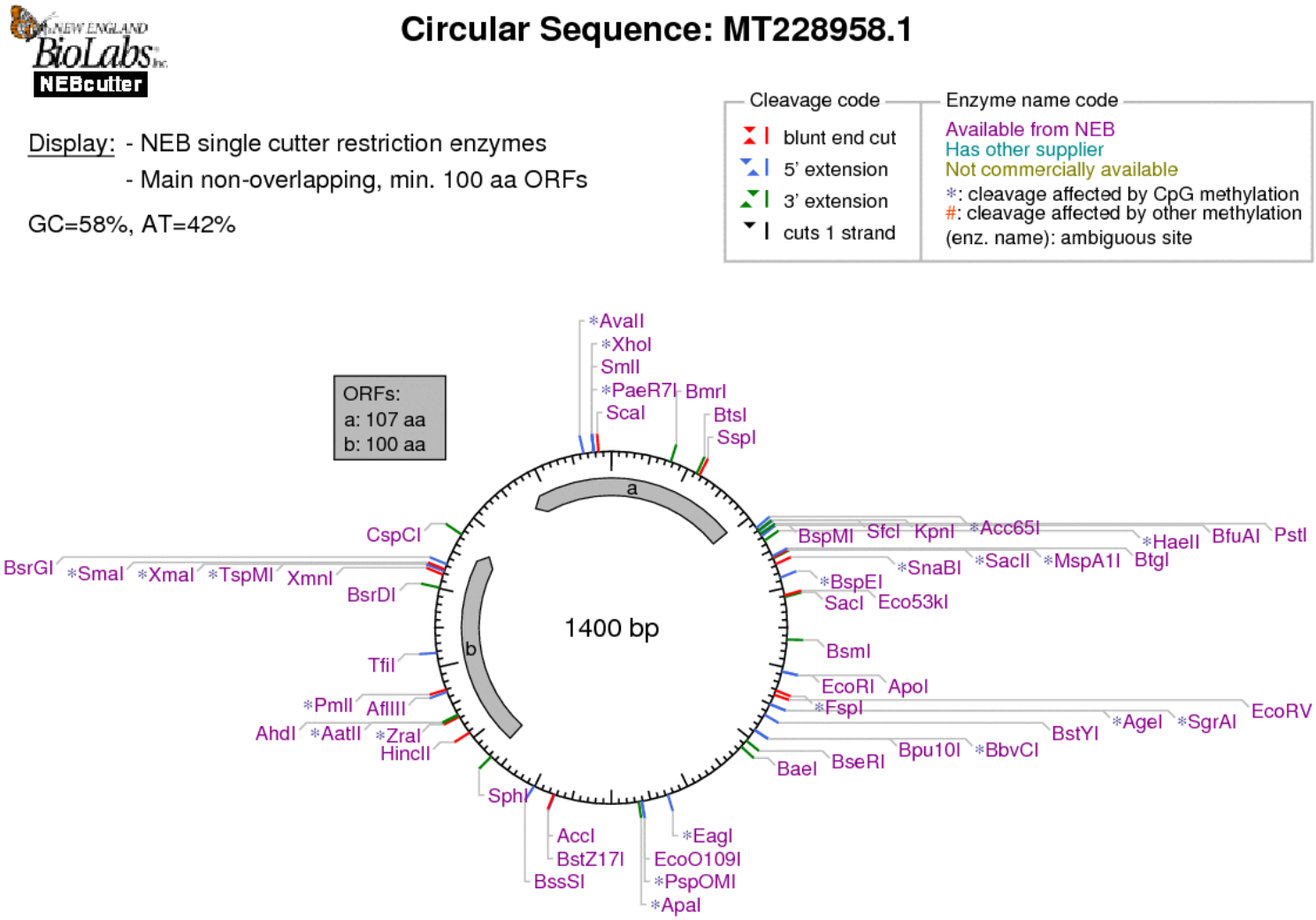

Figure 4

Circular sequence structure with possible restriction sites positions of S. toxytricini D2

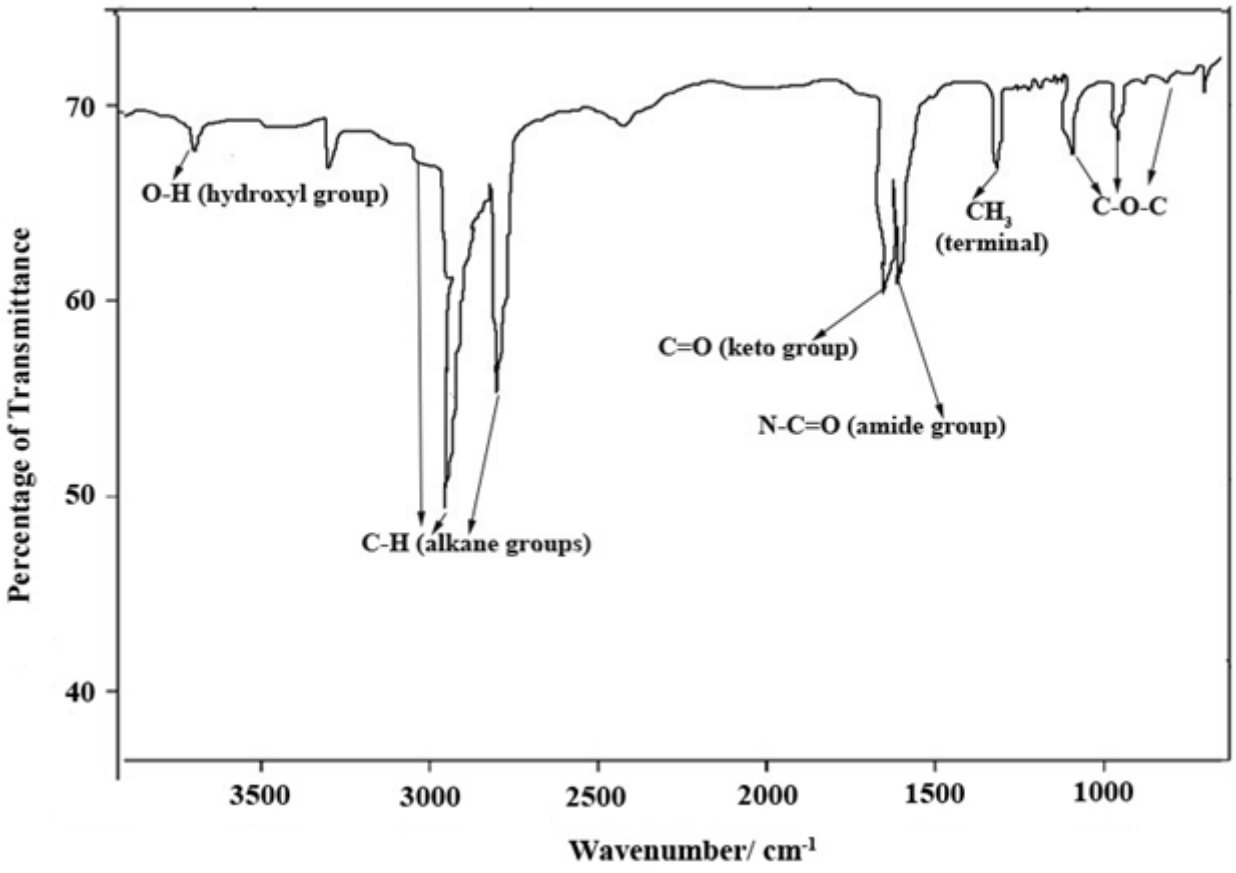

Figure 5

FTIR analysis of PHAs extracted from S. toxytricini D2

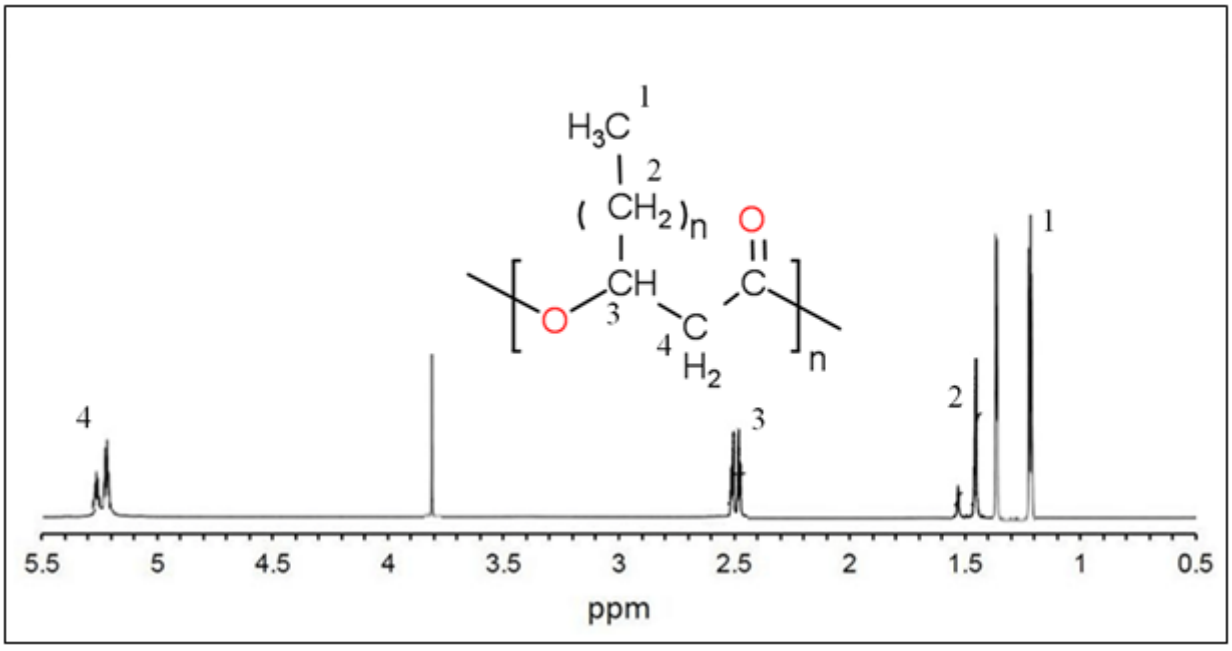

Figure 6

1H NMR analysis of PHAs extracted from S. toxytricini D2

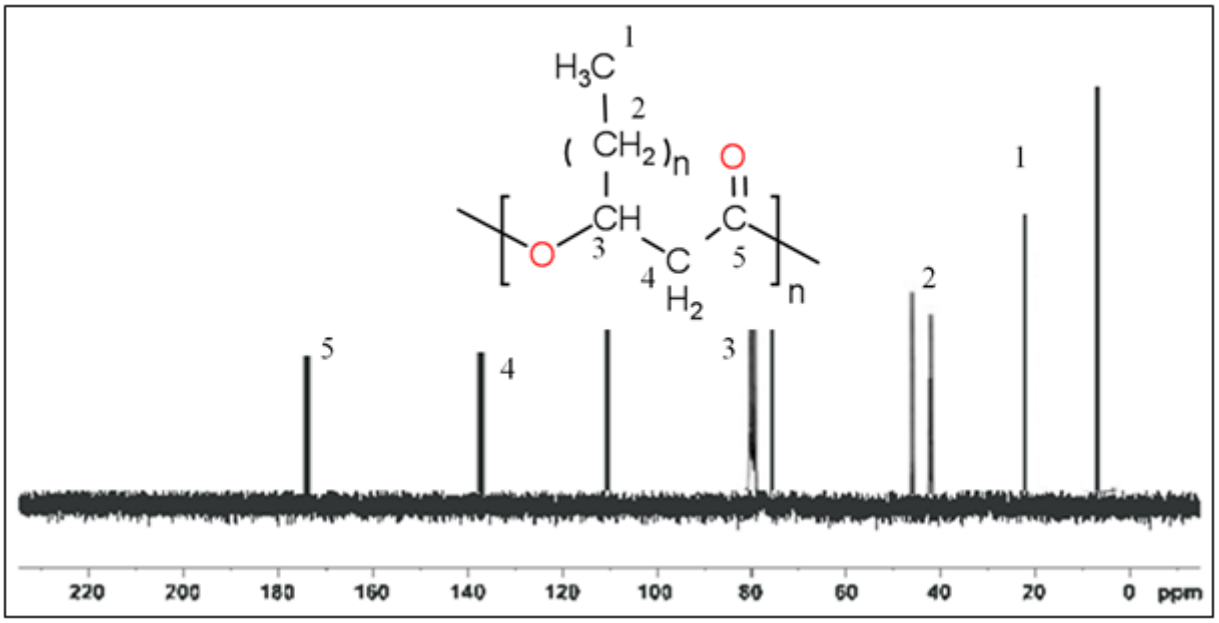

Figure 7

13C NMR analysis of PHAs extracted from S. toxytricini D2

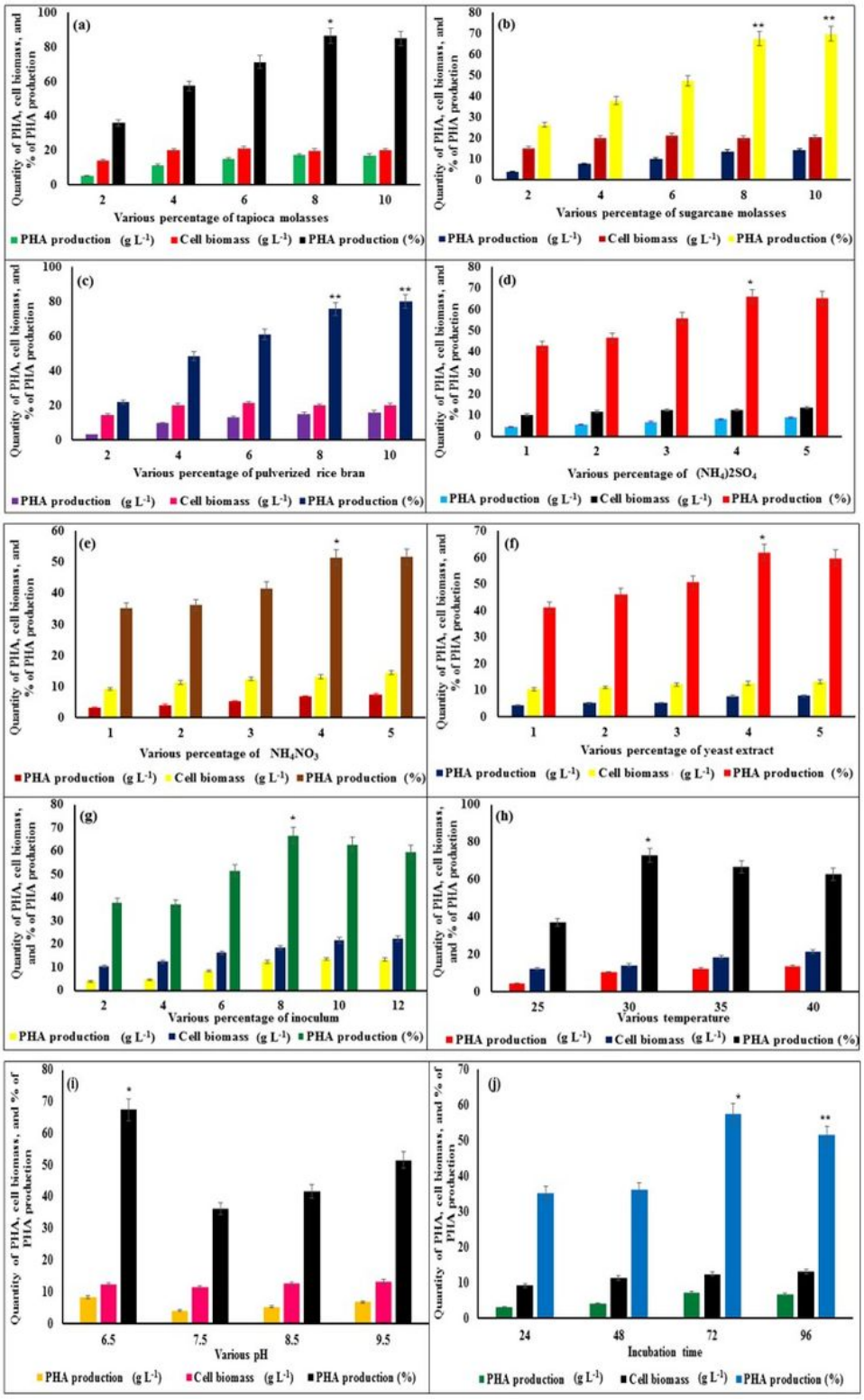

Figure 8

Growth parameters optimization for PHAs fabrication by S. toxytricini D2

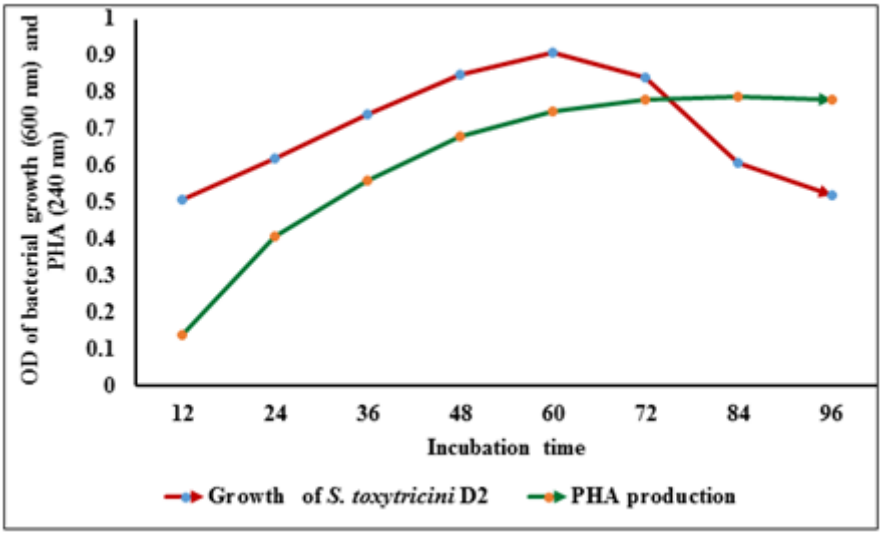

Figure 9

Growth kinetics and PHAs production under optimized growth conditions