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Poly-3-hydroxyalkanoates production potential of *Bacillus cereus* C113 isolated from cassava dumpsite using some carbon sources

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Abstract

Poly-3-hydroxyalkanoates (PHAs) are biopolymers accumulated as intracellular energy reserves by bacteria under nutrient limiting growth conditions and suitable for plastic production. We report findings from the use of a bacterium isolated from a cassava dumpsite for PHAs production. Isolate C113 was screened for PHAs production using the viable colony and Sudan Black B staining methods and further identified by 16SrRNA sequencing. Its PHA synthase gene, *PhaR*, was also partially amplified and sequenced. PHAs production was achieved over 96-hour incubation, extracted by NaClO/chloroform method and analysed by FT-IR. Isolate C113 was positive for PHA production in all carbon sources and was identified as a strain of *Bacillus cereus*. It showed highest biomass accumulation in glucose and starch. It achieved PHAs production of 4.85 % dry cell weight (DCW) in glucose, 33.03 %DCW in glycerol and 6.05 %DCW in sugarcane molasses at 24 hours while 10.58 %DCW was produced in starch at 96 hours. FT-IR spectra showed peaks corresponding to P3HB and P3HB3HV and reveal conformational changes of mcl-PHA and scl-PHA in crystalline and amorphous phases. The 16SrRNA (KY855372.1) and *PhaR* (MF947451.1) sequences have been accessioned in NCBI-GenBank. The results show that *Bacillus cereus* C113 demonstrated capacity to utilize a variety of carbon sources for PHAs production.

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Introduction

Poly-3-hydroxyalkanoates (PHAs) are biodegradable elastomers that show valuable material properties (Spiekermann et al., 1999; Babruwad et al., 2015). They are made as intracellular carbon inclusions by bacteria under conditions of limiting nitrogen and phosphorous in a carbon reach medium (Salaam et al., 2017). Thus, they can be used in bio-plastic production of commercial value comparable with petrochemical plastics (Alkotaini et al., 2015). PHAs are classified into three major types namely: small chain length (SCL) containing 3 – 5 carbon atoms, medium chain length (MCL) containing 6 – 14 carbon atoms and long chain length (LCL) containing ≥ 15 carbon atoms (Rehm and Steinbüchel, 1999; Tan et al., 2014).

The PHA biosynthesis genes are clustered into the *phaCAB* operon in bacteria comprising of *PhaA, PhaB* and *PhaC* genes which code for β-ketothiolase, NADP-dependent acetoacetyl-CoA reductase and PHA synthase respectively (Rehm and Steinbüchel, 1999). The PHA synthases produced by various bacteria are classified into four. Class I synthases are made by *Ralstonia eutropha*, class II by *Pseudomonas,* class III by *Allochromatium venosum* and class IV by *Bacillus* sp (Ali and Jamil, 2016). The class IV synthases are encoded by two genes, *PhaC* and *PhaR,* and both are important for PHA polymerization

(McCool and Cannon, 2001).

A major limitation in PHAs production is the cost of carbon source which has been reported to account for up to 40 - 60% of total production cost (Du et al., 2012; Fadipe et al., 2018). Hence, there is need to explore cheap carbon sources as potential substrates for PHAs production. Carbon rich agro-industrial wastes such sugarcane molasses, crude glycerol and cassava processing wastes are potential candidates (Gomaa, 2014; Aro et al., 2016). Utilization of these wastes will be beneficial in waste management as well as economic commercialization of PHAs production (Aremu et al., 2010).

Bacillus sp are well known bacterial species with ability to utilize varieties of complex carbon sources for growth (Alkotaini et al., 2015). The use of some members of this group in PHAs production has been reported by several authors. Examples of members of the group applied in PHAs production are *B. cereus, B. thrungiensis, B. subtilis* and *B. megaterium, B. mycoides* (Wu et al., 2001; Aarthi and Ramana, 2011; Ali and Jamil, 2014). Strains of *B. cereus* have demonstrated capacity for PHAs production including *B. cereus* SPV, *B. cereus* PW3A (Akaraonye et al., 2012), *B. cereus* DC1, B. cereus DC2, B. cereus DC3, B. cereus DC4 (Aarthi and Ramana, 2011) and *B. cereus* PS-10 (Sharma and Bajaj, 2015).

They have been shown to utilize glucose, fructose, galactose and sucrose as carbon sources for PHAs production (Babruwad et al., 2015). They have also utilized a wide variety of low-cost sustainable carbon substrate such as maize bran, rice husk, wood waste, sugarcane molasses, glycerol, almond shell powder, walnut shell powder, wheat starch, soluble starch, corn starch, potato starch for PHAs production (Aarthi and Ramana, 2011; Sharma and Bajaj, 2015). B. cereus PW3A produced 0.325 g/l of polyhydroxybutyrate (PHB) using 5% w/v glucose as carbon source as reported by Babruwad et al (2015). *B. cereus* PS-10 utilized 3% w/v molasses and 2% v/v glycerol as carbon substrates to produce 9.5 g/l and 8.9 g/l PHB as reported by Sharma and Bajaj (2015). Our study reports the use of a new strain of *Bacillus cereus* isolated from cassava dumpsite with potential for sustainable PHAs production.

Materials and Methods

Screening of Isolate C113 for PHAs Production

Bacterial strain C113 previously isolated from a cassava dumpsite by Salaam et al (2017) was obtained from the Culture Collection Center at the Biotechnology Department of the Federal Institute of Industrial Oshodi, Lagos, Nigeria. It was screened for PHA production using 2 % w/v carbon source (glucose, glycerol (v/v), sugarcane molasses and starch) by the viable colony staining method using Nile red and Nile blue A. Isolate C113 was cultured for 24 – 48 hours on PHA detection agar (PDA) containing 15 g/l agar,13.30 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 1.2 g/l $MgSO_4 \cdot 7H_2O$, 1.7 g/l citric acid and 10 ml trace elements solution (4.5 g/l $ZnSO_4.7H_2O$, 0.7 g/l $MnCl_2.4H_2O$, 0.2 g/l $CuSO_4.2H_2O$, 0.4 g/l $Na_2MoO_4.2H_2O$, 4.5 g/l $CaCl_2.2H_2O$ and 3 g/l $FeSO_4.7H_2O$), supplemented with 0.5 µg ml^{-1} of Nile Blue A and Nile Red. Plates were visualized under ultraviolet light (260 – 350nm) for fluorescence detection of PHA (Spiekermann et al., 1999). Further screening was also carried out by Sudan black B staining for the intracellular detection of PHA granules (Salaam et al., 2017). The slides were observed under the light microscope for blue black intracellular bodies and photomicrographs were taken for documentation.

Molecular Characterization of Isolate`C113

Deoxyribonucleic acid was isolated from pure culture of isolate C113 grown in nutrient broth for 16 - 18 hours at 37°C and 120 rpm according to protocol outlined in the GeneAll® Exgene™ Cell SV kit. Amplification of the 16SrRNA and sequencing was carried out at 1st Base (Singapore). Sequence homology was performed using the NCBI-BLASTn tool. Phylogenetics was performed in MEGA 6 using ClustalW alignment tool and Neighbour-Joining algorithm considering 1000 bootstrap replications.

Amplification of Class IV PhaR Gene

Gene specific primers for the PHA synthase gene, *PhaR* were designed using the Primer3Plus software. The partial coding sequence of the *PhaR* gene was amplified using the designed primer pair: PhaRBcF, CGAAACATTTTGGGGAAAAG and PhaRBcR, TCGTAACATCGCGCTTTAAT. The reaction

mixture of 50 µl contained 25 µl Thermo Scientific Dream Taq Green PCR Master mix, 19 µl nuclease free water, 0.5 µl each of 0.1 mM forward and reverse primers and 5 µl template DNA. Cycling conditions consisted of initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 55.71°Cfor 1 minute, extension at 72°C for 1 minute, final extension at 72°C for 5 minutes and hold at 4°C in a Kyratec Supercycler Trinity. The 281 bp PCR amplicon was sequenced at Macrogen (Korea). Sequence homology was performed using the NCBI-BLASTn tool. Phylogenetics was done in MEGA 6 using ClustalW alignment and Neighbour-Joining algorithm with 1000 bootstrap replications.

Production of PHA by Isolate C113

Seed culture was prepared by growing pure culture of isolate C113 in 50 ml nutrient broth for 16 - 18 hours at 37°C and 120 rpm. The broth culture was centrifuged for 10 minutes at 6000 rpm, washed with nitrogen limiting mineral salt medium (NL-MSM) containing 13.3 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$ 1.2 g/l $MgSO_4.7H_2O$ and 1.7 g/l citric acid with 10 ml trace elements solution (4.5 g/l $ZnSO_4.7H_2O$, 0.7 g/l $MnCl_2.4H_2O$, 0.2 g/l $CuSO_4.2H_2O$, 0.4 g/l $Na_2MoO_4.2H_2O$, 4.5 g/l $CaCl_2.2H_2O$ and 3 g/l $FeSO_4.7H_2O$). Then re-centrifuged at 6000 rpm for 7 minutes, the resulting pellet was dissolved in 5 ml of NL-MSM (Ali and Jamil, 2016). The re-constituted pellet was inoculated (optical density 0.05 - 0.1 at 600 nm) into 300 ml of NL-MSM supplemented with a 2 % w/v carbon substrate (glucose; glycerol (v/v); sugarcane molasses; starch). The flasks were incubated for 96 hours at 37°C and 120 rpm. Samples (50 ml) from the incubated flasks were drawn at 24 hours interval for PHA extraction. Optical density at 600 nm was noted over the incubation period and used to estimate its growth curve. All cultures were transferred under aseptic conditions and grown in duplicates.

Extraction and Characterization of PHA

Extraction of PHA was according to modifications of Ali and Jamil (2016) and Berger et al (1989) methods. Twenty-four hourly cultures (50 ml) drawn from the production medium above was centrifuged at 6500 rpm for 10 minutes and the bacterial pellet was lyophilized to get the dry cell weight (DCW). The lyophilized biomass was treated with 10 ml of freshly prepared 5.25 % NaClO (v/v), pH 10 and incubated at 120 rpm and 37°C for 40 minutes. The mixture was transferred into conical flask containing 30 - 50 ml of chloroform and incubated at 120 rpm and 37°C for 100 minutes. The biomass-hypochlorite-chloroform mixture was transferred unto a glass separating funnel to obtain the lower dense phase of chloroform that contained the extracted PHA. The chloroform-PHA mixture was concentrated and chloroform recovered in an Heidolph 16-G1B diagonal condenser rotary evaporator. The concentrated mixture was dried at room temperature and the weight of the extracted PHA was determined and the percentage PHA content of the DCW was calculated using the formula below:

$$Extracted\ PHA\ (\frac{g}{l}) \div DCW\ \times 100$$

Where, g is the weight of extracted PHA in grams and l is one litre of PHA production medium.

For Fourier Transform Infrared Spectroscopy (FT-IR), 10 mg of extracted PHA was treated with potassium bromide (KBr) and analyzed for the presence of functional groups by an Agilent Cary 660 FT-IR spectrophotometer with spectral range of 650 cm^{-1} – 4000 cm^{-1}, resolution of 2 cm^{-1} and 32 - 64 scans averaged. The FT-IR spectra were acquired using the KBr disc method (Ali and Jamil, 2016).

Results and Discussion

Isolate C113 produced orange and yellow fluorescence under UV light with Nile Red (NR) and Nile Blue A (NBA) respectively in the various carbon sources (Fig. 1A). The orange colour indicates the accumulation of polyhydroxybutyrate while the yellow fluorescence indicates the presence of co-polymers (Spiekermann et al., 1999). The presence of blue-black intracellular inclusions of PHAs (Fig. 1B) were also seen with Sudan Black B staining for the four carbon sources which is confirmatory for the accumulation of PHAs (Salaam et al., 2017).

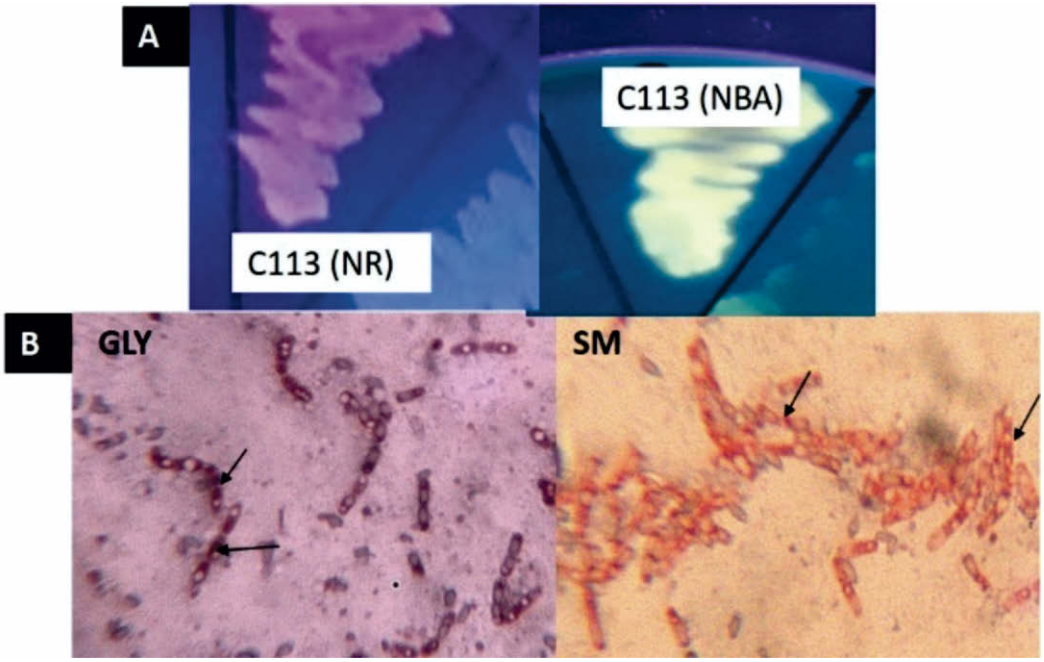

Figure 1: Fluorescence (A) and Intracellular (B) Detections of PHAs in Isolate C113. NR – Nile Red, NBA – Nile Blue A, GLY - Glycerol, SM – Sugarcane Molasses. Black arrows indicate cells with intracellular PHA inclusions.

Following 16SrRNA sequencing, the organism was identified as *Bacillus cereus* strain C113 (NCBI Accession number: KY855372.1) sharing closest homology with *B. cereus* JCM 2152 at 99%. *Bacillus cereus* C113 clusters perfectly with other B. cereus strains as shown in the neighbor-joining tree below.

(Fig. 2). Several strains of *B. cereus* group isolated from wide variety of habitats are notable for PHAs production ability (Aarthi and Ramana, 2011; Akaraonye et al., 2012; Sharma and Bajaj, 2015).

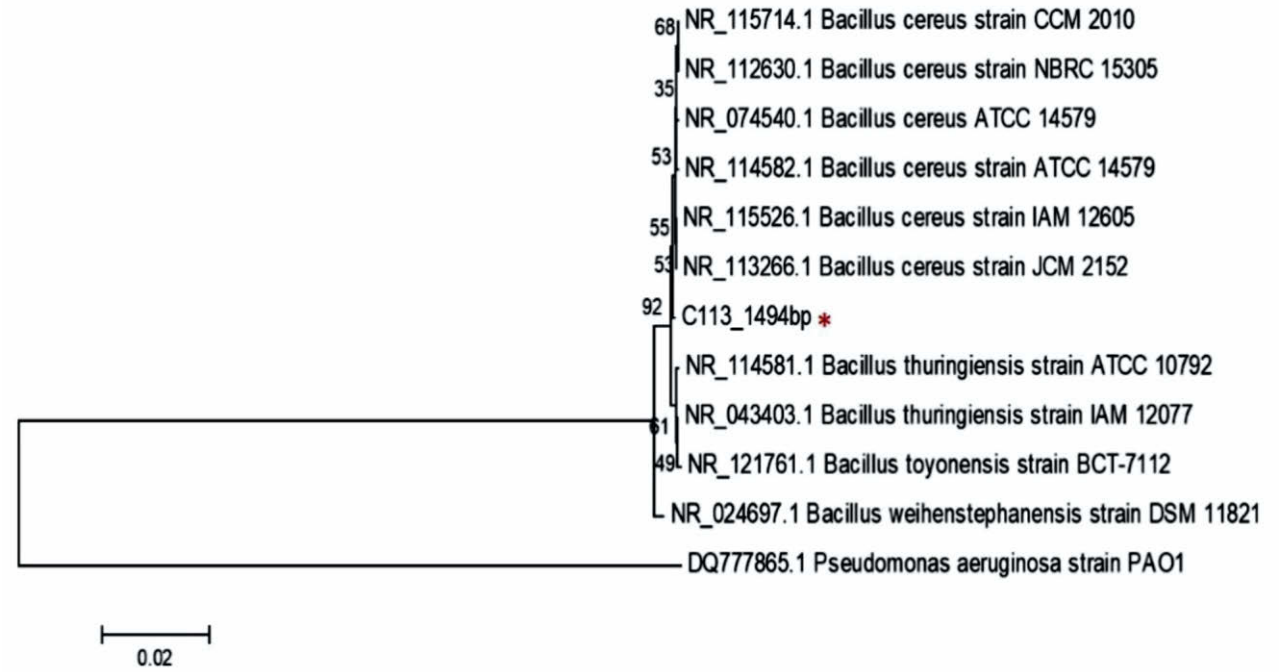

Figure 2: Phylogenetic Tree of 16SrRNA Sequences of *Bacillus cereus* C113rooted to *Pseudomonas aeruginosa*. *B. cereus* C113 is indicated by the red asterisk

The successful amplification of the 281 bp fragment of the *PhaR* gene in *B. cereus* C113 indicates that it belongs to the class IV PHAs producers (Fig. 3). Sequence homology revealed that *B. cereus* C113 shared 99 % homology with *B. cereus* YB4 and phylogenetic analysis of the predicted *PhaR* protein also showed *B. cereus* C113 clustered with *B. cereus* YB4 (Fig. 4). The nucleotide sequences have been submitted and accessioned in the NCBI-GenBank (MF947451.1). The *Bacillus* genera have been classified as class IV PHAs organisms because it possesses the *PhaR* gene in addition to the *PhaC* gene (codes for the functional PHA synthase) (Rehm and Steinbüchel, 1999; Sharma and Bajaj, 2015).

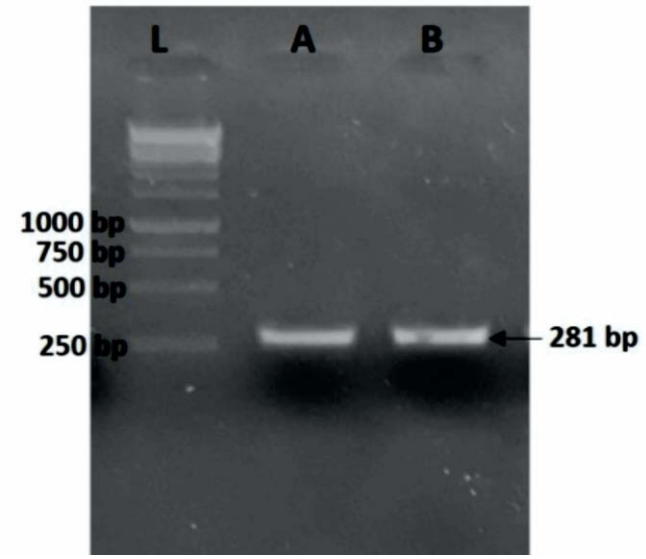

Figure 3: PCR Amplicon Agarose Gel of PhaR Gene. L – 1 kb DNA Ladder, A and B – PCR amplicons in duplicate

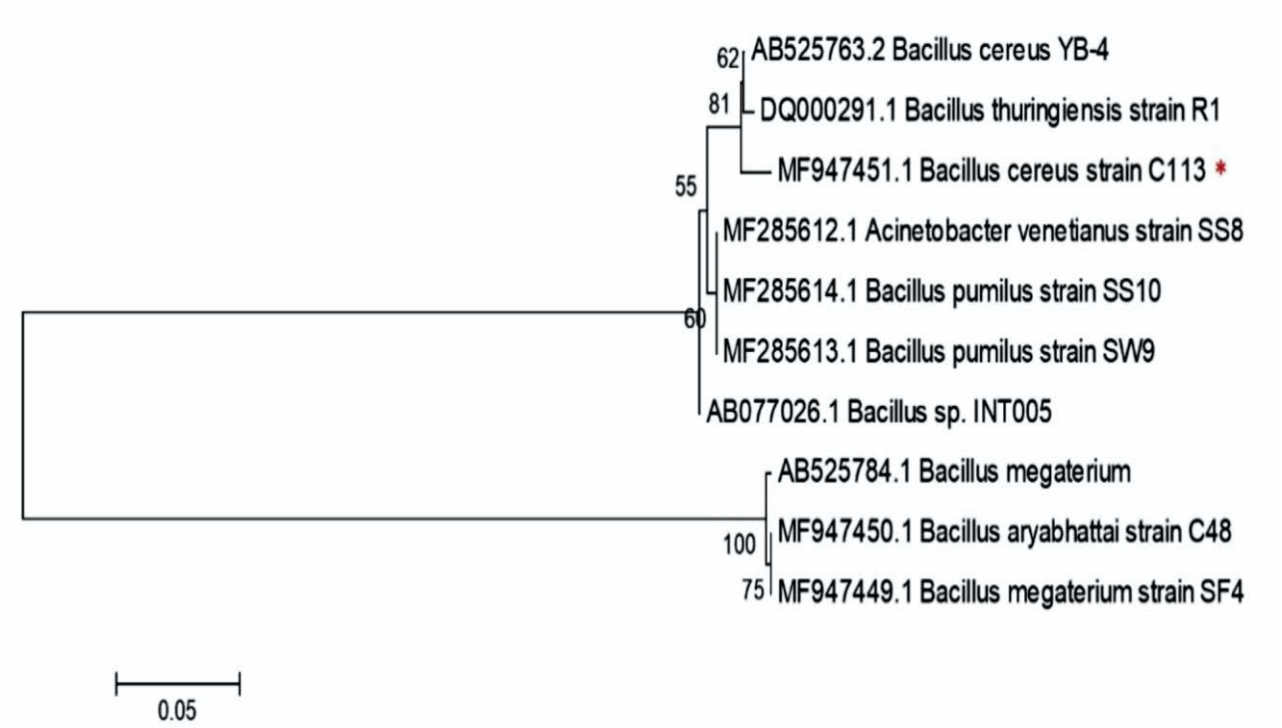

Figure 4: Phylogenetic Tree of Predicted *PhaR* Protein of *Bacillus cereus* C113, *B. cereus* C113 is indicated by the red asterisk.

The growth curve showed that B. cereus C113 utilized glucose and starch as the best carbon sources (Fig. 5A). Thus, it has potential for utilization of starch from agro-industrial wastes for PHAs production. In 2 % starch, 2 % glucose and 2 % glycerol supplemented NL-MSM, *B. cereus* C113 grew exponentially and peaked at 24 hours after which another prominent peak was observed at 48 hours. Afterward, its growth continued to increase steadily over the 96-hour incubation period. In 2 % sugarcane molasses supplemented NL-MSM, its growth increased exponentially and peaked at 24 hours followed by a steady increase in growth with a possible decline beginning after the 96-hour incubation period. Our findings agree with previous work which showed 24 – 48 hours as the time of exponential growth of *B. cereus* strains in glucose, glycerol, starch and sugarcane molasses (Ali and Jamil 2014; Babruwad et al., 2015; Sharma and Bajaj, 2015).The utilization of carbon sources by *B. cereus* C113 can be summarized as glucose > starch > glycerol > sugarcane molasses.

In Fig. 5B, it also achieved the highest biomass (expressed as dry cell weight, DCW) accumulation in these two carbon sources. Our results showed that significant biomass was produced by *B. cereus* C113 in the four carbon sources at 24- and 48-hours incubation time which has been reported as the best incubation time for PHA accumulation by members of the *Bacillus* genera and some strains of *B. cereus* (Akaraonye et al., 2012). *B. cereus* C113 achieved biomass accumulation of 2.04 ± 0.25 g/l and 6.6 ± 0.28 g/l (highest biomass accumulation in our study) in glucose at 24 hours and 48 hours respectively. At 24- and 48-hours incubation time in glycerol, it achieved biomass of 0.67 ± 0.31 g/l and 3.52 ± 0.51 g/l respectively. In starch, we recorded biomass of 1.52 ± 0.20 g/l at 24 hours and 3.59 ± 0.95 g/l at 48 hours. In sugarcane molasses it also produced biomass 2.79 ± 0.33 g/l and 4.43 ± 0.13 g/l after 24- and 48-hours incubation respectively.

Our results agree with findings that B. cereus strains, which are gram positive bacteria, produce biomass significant for PHA in the exponential growth phase during production. Therefore, cultivation techniques to improve biomass accumulation in the exponential growth phase is recommended for the *Bacillus* genera (Aarthi and Ramana, 2011; Sharma and Bajaj, 2015). Therefore, cultivation techniqueswhich will improvebiomass accumulation of B. cereus C113 in its exponential growth phase are worthy of further investigation.

B. cereus C113 achieved PHA production of 4.85 % DCW in glucose, 33.03 % DCW in glycerol and 6.05 % DCW in sugarcane molasses at 24 hours while 10.58 % DCW was produced in starch at 96 hours (Fig. 5C). In another reported study, *B. cereus* 64-INS achieved 64.5 % DCW in glucose and 16.59 % DCW in sugarcane molasses (Ali and Jamil 2014). *B. cereus* SPV utlized sugacane molasses in a 1 litre shake flask experiemnt to produce 61.07 % DCW of PHA (Akaraonye et al., 2012). The reduced PHA yield we report could be an outcome of incomplete digestion of bacterial biomass or degradation of PHA during NaClO treatment. Sodium hypochlorite (NaClO) has been demonstrated to cause serious degradations of PHA subsequently leading to up to 50 % decrease in yield (Wu et al., 2001; Tan et al., 2014). Nevertheless, a heat or freezing pretreatment prior to sodium hypochlorite digestion has been shown to increase PHA by up to 98 % (Akaraonye et al., 2012). Another factor could be the lower concentration of 5.25 % NaClO used in our study in contrast to 13 % and 70 % previously reported by Ali and Jamil (2014) and Akaraonye et al (2012) respectively. In addition, equal amount ratio was maintained between NaClO and chloroform during the extraction used in other studies (Akaraonye et al., 2012; Ali and Jamil, 2014). The effects of these treatments, increase in NaClO concentration and the use of an equal ratio of NaClO and chloroform during PHA extraction from *B. cereus* C113 biomass is worthy of further investgations.

Furthermore, examination of Sudan Black B stained cells revealed that complete spores were formed by *B. cereus* C113 as incubation time increased beyond 48 hours. This suggests sporulation as a mechanism by which it adapts to the imbalanced growth conditions thus causing reduced PHA yield. This phenomenon has been reported in some spore forming *Bacilli* during PHA production (Nicolas et al., 2008). This sporulation phenomenon during PHA production by spore forming *B. cereus* has been succesfully prevented by adding up 90 mM α-picolinic

acid or maintaining low pH during production (Akaraonye et al., 2012).We also report 0.1 g/l PHA using 2 % glucose at 37°C after 48 hours incubation while *B. cereus* PW3A used 5 % glucose to produce 0.325 g/l PHB at 35°C after 48 hours incubation as reported by Babruwad et al (2015). This difference in PHA amount may be due to the higher amount of carbon source supplied for conversion in the exponential phase of bacterial growth.

In addition, we report that *B. cereus* C113 utilized 2 % molasses and 2 % glycerol to 0.17 ± 0.04 g/l and 0.22 ± 0.03 g/l PHA respectively. However in another study with *B. cereus* PS -10 using 3 % molasses and 2 % glycerol, 9.5 g/l and 8.95 g/l PHB respectively was produced as reported by Sharma and Bajaj (2015). The huge difference in PHA yield may be due to the starting inoculum size of 0.9 at absorbance of 600nm used with *B. cereus* PS -10 compared to our smaller inoculum size of 0.05 – 0.1 at absorbance of 600 nm. This is significant and corroborated by earlier observation from previous works that showed members of the *Bacillus* genera to accumulate actively PHA during the growth phase when nutrients are more abundant in the production medium (Aarthi and Ramana, 2011). Therefore, starting the experiment with more inoculum is expected to lead to more PHA accumulation during the early growth and up to the end of the exponential growth phase in *Bacillus* species. It will be interesting to investigate the optimum inoculum size and carbon source amount for maximum PHA production by *B. cereus* C113.

Analyses of the four FT-IR spectra of extracted PHA (Fig. 6 A - D) produced using glucose (A), glycerol (B), sugarcane molasses (C) and starch (D) below showed peaks indicative of PHB and PHB-HV co-polymer accumulations. Furthermore, they reveal conformational changes of mcl-PHA and scl-PHA in crystalline and amorphous phases. Members of the *Bacillus* group have been previously reported as producers of scl-PHAs utilizing various carbon sources (Song et al., 2008; Gumel et al., 2012; Getachew and Woldesenbet, 2016). Peaks at 2923 cm^{-1} (Fig. 6A), 2929 cm^{-1} and 2925 cm^{-1} (Fig. 6B), 2916 cm^{-1} (Fig. 6C) and 2920 cm^{-1} (Fig. 6D) correspond to asymmetric stretching vibration in CH_2 lateral chains. Further peaks at 2853 cm^{-1} (Fig. 6A), 2854 cm^{-1} (Fig. 6B), 2849 cm^{-1} (Fig. 6C) and 2851 cm^{-1} (Fig. 6D) represent

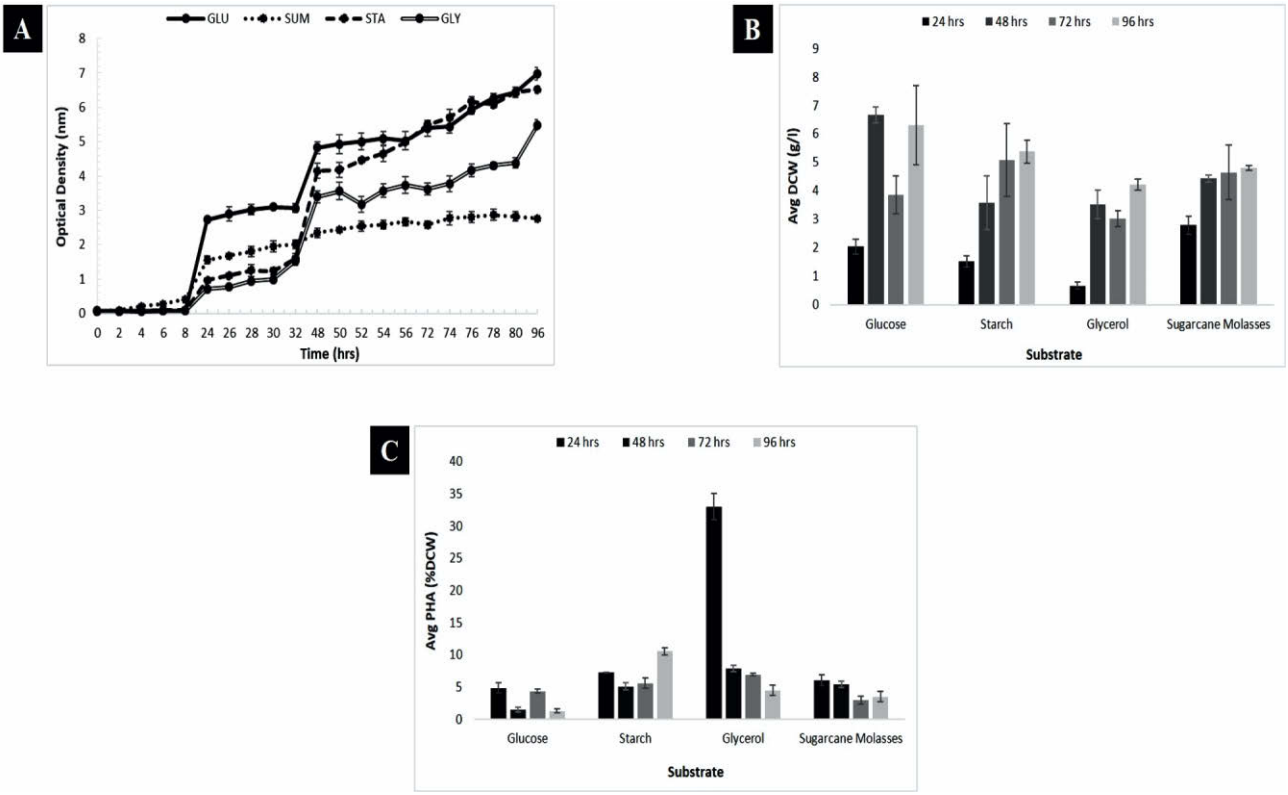

Figure 5: Growth Curve, Dry Cell Weight of and PHA Extracted from Bacillus cereus C113. GLU – glucose, GLY - glycerol, SUM – sugarcane molasses, STA – starch, DCW - dry cell weight.

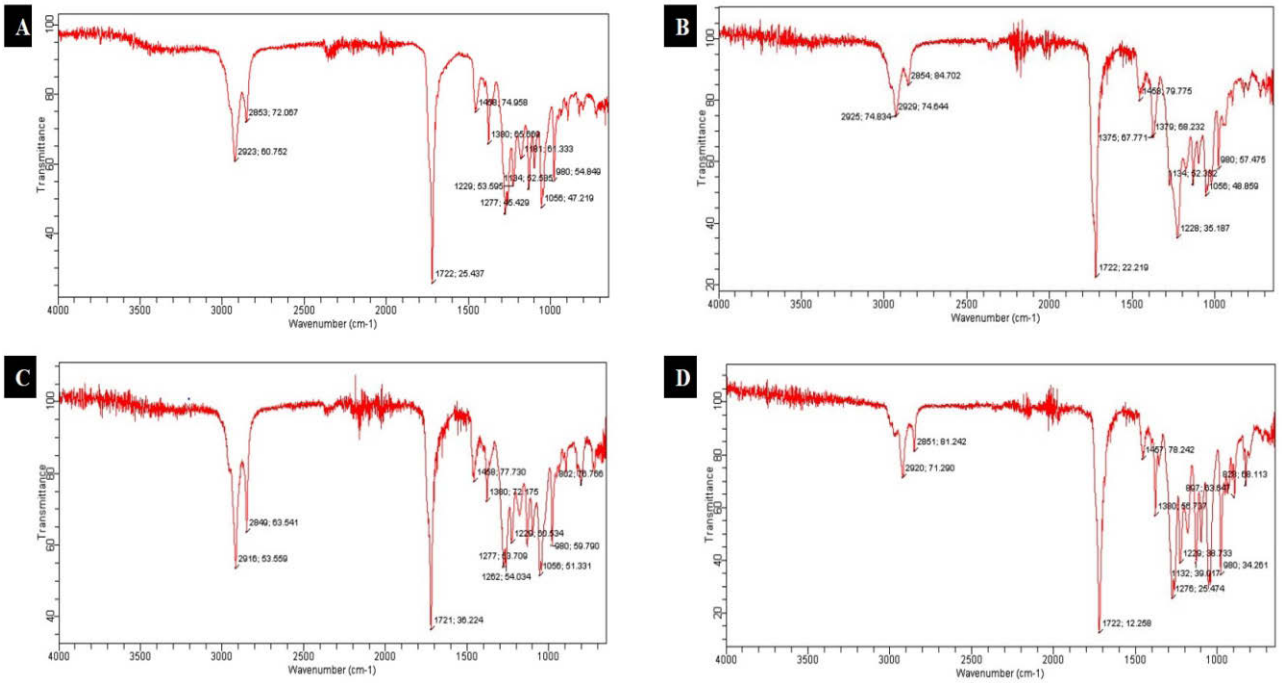

Figure 6: FTIR Spectra of Extracted PHA Produced by Bacillus cereus C113 using Four Carbon Substrates. A – glucose, B – glycerol, C – sugarcane molasses, D – soluble starch.

symmetrical CH_3 groups and are formed due to conformational changes during crystallization (Sharma and Bajaj, 2015).

In addition, peaks at 1722 cm^{-1} (Figs. 6A - B and 6D) and 1721 cm^{-1} (Fig. 6C) are rotations around carbon atoms of ester carbonyl groups in chains of ordered crystalline structures and are PHA marker bands (Babruwad et al., 2015). Absorption peaks at 1458 cm^{-1} (Figs. 6A – 6C) and 1457 cm^{-1} (Fig. 6D) are bacterial intracellular amide II (Getachew and Woldesenbet, 2016). Peaks at 1380 cm^{-1} (Figs. 6A and 6C - D), 1379 cm^{-1} and 1375 cm^{-1} (Fig. 6B) are terminal CH_3 groups (Kumalaningsih et al., 2011). Peaks at 1277 cm^{-1} (Figs. 6A and 6C), 1262 cm^{-1} (Fig. 6C) and 1276 cm^{-1} (Fig. 6D) correspond to C-O-C stretching vibrations (Getachew and Woldesenbet, 2016). Peaks at 1229 cm^{-1} (Figs. 6A and 6C - D) and 1228 cm^{-1} (Fig. 6B) represent asymmetric -C-O- bonds (Kumalaningsih et al., 2011). Peaks at 1181 cm^{-1} (Fig. 6A), 1134 cm^{-1} (Figs. 6A and 6B), 1056 cm^{-1} (Figs. 6A - 6C) and 1132 cm^{-1} (Fig. 6D) are C–O stretching vibrations in amorphous phase (Gumel et al., 2012). Other absorption peaks at 980 cm^{-1} (Figs. 6A – 6D), 802 cm^{-1} (Fig. 6C), 897 cm^{-1} and 828 cm^{-1} (Fig. 6D) suggest the occurrence of alkyl halides in the extracted polymers (Getachew and Woldesenbet, 2016).

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