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Enhanced Agarose and Xylan Degradation for Production of Polyhydroxyalkanoates by Co-Culture of Marine Bacterium, *Saccharophagus degradans* and Its Contaminant, *Bacillus cereus*

Shailesh S. Sawant [1], Bipinchandra K. Salunke [1], Larry E. Taylor II [2] and Beom Soo Kim [1,*]

[1] Department of Chemical Engineering, Chungbuk National University, Cheongju, Chungbuk 28644, Korea; shailesh.nccs@gmail.com (S.S.S.); bipinchandra.salunke@gmail.com (B.K.S.)

[2] Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA; larry.taylor@nrel.gov

* Correspondence: bskim@chungbuk.ac.kr; Tel.: +82-43-261-2372; Fax: +82-43-269-2370

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Abstract: Over reliance on energy or petroleum products has raised concerns both in regards to the depletion of their associated natural resources as well as their increasing costs. Bioplastics derived from microbes are emerging as promising alternatives to fossil fuel derived petroleum plastics. The development of a simple and eco-friendly strategy for bioplastic production with high productivity and yield, which is produced in a cost effective manner utilising abundantly available renewable carbon sources, would have the potential to result in an inexhaustible global energy source. Here we report the biosynthesis of bioplastic polyhydroxyalkanoates (PHAs) in pure cultures of marine bacterium, *Saccharophagus degradans* 2-40 (*Sde* 2-40), its contaminant, *Bacillus cereus*, and a co-culture of these bacteria (*Sde* 2-40 and *B. cereus*) degrading plant and algae derived complex polysaccharides. *Sde* 2-40 degraded the complex polysaccharides agarose and xylan as sole carbon sources for biosynthesis of PHAs. The ability of *Sde* 2-40 to degrade agarose increased after co-culturing with *B. cereus*. The association of *Sde* 2-40 with *B. cereus* resulted in increased cell growth and higher PHA production (34.5% of dry cell weight) from xylan as a carbon source in comparison to *Sde* 2-40 alone (22.7% of dry cell weight). The present study offers an innovative prototype for production of PHA through consolidated bioprocessing of complex carbon sources by pure and co-culture of microorganisms.

Keywords: polyhydroxyalkanoates; agarose; xylan; *Saccharophagus degradans*; *Bacillus cereus*; consolidated bioprocessing

1. Introduction

Saccharophagus degradans 2-40 (*Sde* 2-40; early name *Microbulbifer degradans* 2-40), a motile gamma proteobacterium of marine origin, uniquely utilises different carbon sources and degrades a variety of complex polysaccharides such as agar, alginic acid, carrageenan, cellulose, chitin, glucan, laminarin, pectin, pullulan, starch, and xylans [1–6]. *Sde* 2-40 can powerfully degrade polysaccharides from plant and marine sources. Agarose, the main agar constituent, is acquired from marine algae (Rhodophyta) such as *Gelidium* and *Gracilaria* [7,8]. Xylan, the principal constituent of plant cell wall hemicellulose, is copiously occurring polysaccharide next to cellulose [9]. *Sde* 2-40 has the capability of utilizing agarose and xylan for growth [1,4,6].

Different microorganisms under stress conditions of growth can natively produce carbon storage polyesters like polyhydroxyalkanoates (PHAs) [10,11]. Poly(3-hydroxyburyrate) (PHB) is the most commonly produced PHA by bacteria. *Sde* 2-40 can produce PHB homopolymer using glucose

and starch as carbon sources in marine sea salt minimal media [12–14]. Recently, research interest has been growing in the bacterial cumulative process for the production of PHA using complex polysaccharides [15,16]. *Sde* 2-40, due to its extraordinary metabolic capacity, is deliberated to be a supremely resourceful bacterium for degradation of polysaccharides. The genome sequence of this bacterium contains open reading frames in excess of 180, which encode for enzymes like agarases, alginases, amylases, cellulases, chitinases, pectinases, and xylanases [5]. The expression and characterization of carbohydrases of *Sde* 2-40 was studied through its cultivation in minimal media containing complex polysaccharides [17]. The crucial enzymes involved in PHA synthesis, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, were identified in the genome of *Sde* 2-40 [18]. Reports are available on the growth of *Sde* 2-40 using 0.2% agarose and xylan [6,17]. However, the ability of *Sde* 2-40 to utilize agarose or xylan in higher quantities in growth media and simultaneously produce PHA has not been investigated yet.

In nature, the majority of biotransformations happen due to combined metabolic pathways of diverse microbes such as the natural fermentation of saps containing sugars, compost piles, mammalian gut, aerobic and anaerobic water zones, human skin, and forest soils [19,20]. Utilization of substrates in co-culture occurs via collective metabolic action of the recognized strains of microbes [21]. Co-cultures have been applied in industrial biotechnology for wastewater treatment, remediation of soil, and the production of biogas [22]. In the food industry, co-cultures are used to produce cheese [23], yoghurt [24], and whisky [25]. Co-cultivation of microorganisms can be more beneficial than using a single microorganism in many situations, as it can increase yield, reduce cost of process via inexpensive substrate utilization [26], and control product quality. Co-culture and mixed culture fermentations have great promise for the development of bio-fuels, bio-energy, and bio-based products.

Different wild type and recombinant microorganisms have been reported to produce PHAs using algal and plant biomass [27–29]. However, biomass hydrolysis pre-treatment before fermentation is a prerequisite in these systems for easy assimilation by microorganisms [30]. Low pH and high temperature conditions are generally used for pre-treatment processes. These conditions can prohibit bacterial growth, produce potentially toxic products, and hinder downstream fermentative processes which can lead to lowering yield and increasing cost of production [31–33]. Therefore, a strategy coupling steps of hydrolysis and fermentation (i.e., consolidated bioprocessing) is a smart approach to produce PHAs using carbon sources derived from algae and plants (Figure 1A). In our previous study, we observed that *Bacillus cereus* (KF801505) grows as a contaminant along with *Sde* 2-40 cultures on sea salt minimal media and produces high amounts of PHAs [34]. We observed that the viability as well as agar degradation capability of *Sde* 2-40 increases while growing with *B. cereus* in agar plate cultures. Our ongoing research on the production of PHAs using complex carbon sources and an observation of enhanced growth in co-culture of *Sde* 2-40 and *B. cereus* motivated us to investigate the ability of these bacteria to produce PHAs using complex carbon sources. This study describes PHA production through consolidated bioprocessing of agarose and xylan by *Sde* 2-40, *B. cereus*, and co-culture of *Sde* 2-40 and *B. cereus* without any prior treatment (Figure 1B).

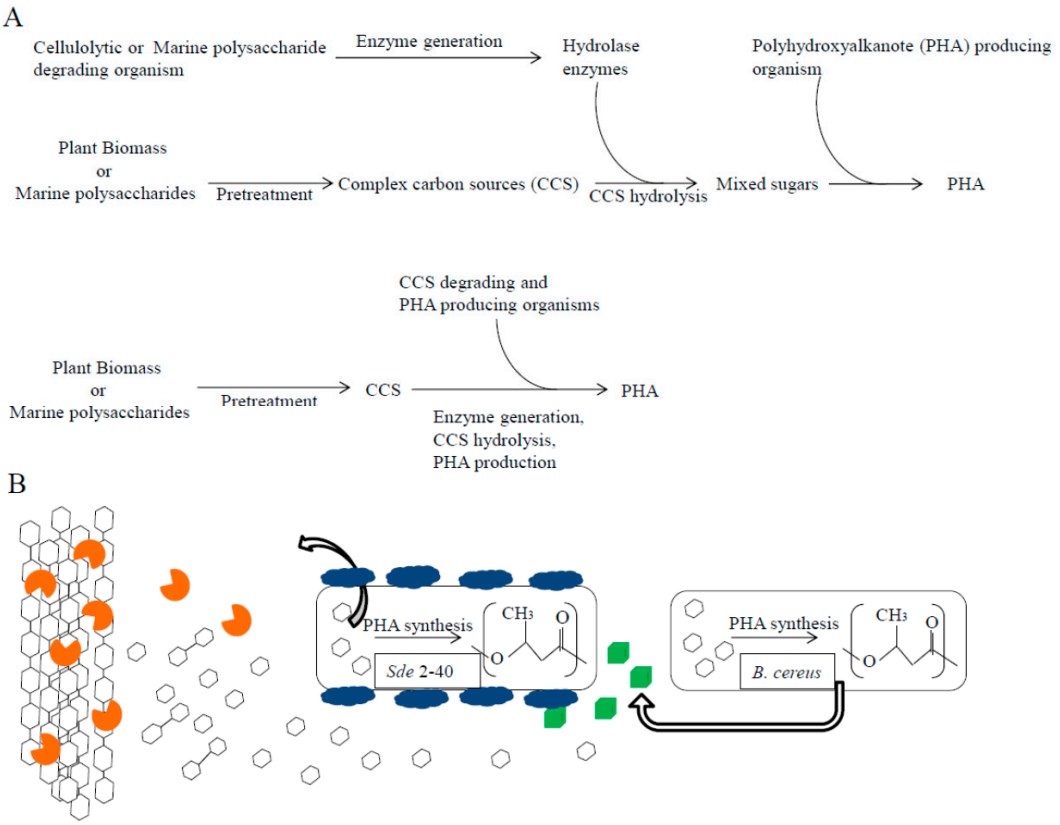

Figure 1. Consolidated bioprocessing of plant biomass or marine polysaccharides to polyhydroxyalkanoates (PHAs) by *Sde* 2-40 and co-culture of *Sde* 2-40 and *B. cereus*. (**A**) Production of PHA by two processes. Classically, sugars are generated from biomass using enzymes with the ability to hydrolyse cellulose and hemicellulose, and PHA is produced by microbes (top). Enzyme production, hydrolysis of biomass, and production of PHA is carried out in single stage in consolidated bioprocessing (bottom); (**B**) Pure and co-culture of microorganisms applied to enhance agarose utilization and PHA production from xylan and agarose. Complex carbon sources are hydrolysed by secreted hydrolase enzymes (orange) to soluble sugars, which are further metabolized to PHA. Cadherin and cadherin-like domains on *Sde* 2-40 cell surface (blue) mediate cell to cell contact. *B. cereus* produce metabolites (green), which may stimulate the growth of *Sde* 2-40.

2. Materials and Methods

2.1. Media

S. degradans 2-40 (ATCC 43961) and *B. cereus* (KF801505) were grown and maintained on either marine agar (half-strength) consisting of 18.7 g/L Marine Broth (Difco, Detroit, MI, USA) supplemented with 1.5% agar (BD, Franklin Lakes, NJ, USA) or minimal media [3] consisting of 23 g/L Instant Ocean Sea salts (Aquarium Systems, Mentor, OH, USA), 1 g/L yeast extract, 50 mM Tris buffer (pH 7.4), 0.5 g/L NH_4Cl, supplemented with 20 g/L glucose, xylan (Beechwood, Sigma, St. Louis, MO, USA), or agarose (Amresco 0710, Solon, OH, USA) as a carbon source at 30 °C in a shaking incubator with speed of 200 rpm.

2.2. Determination of Cell Growth and Dry Cell Weight

The optical density of glucose-grown culture at 600 nm was measured using a spectrophotometer (Shimadzu, Kyoto, Japan). Dry cell weight was determined by centrifugation of the culture at 16,000 rpm for 15 min, two washings of the pellet with 2.3% (*w*/*v*) physiological saline, removal of the supernatant, and vacuum-drying of the cell pellet until constant weight. The insoluble substrates used in this study (agarose and xylan) interfered with the spectrophotometric measurement of the optical density. Therefore, xylan or agarose-grown cell mass was indirectly determined on the basis of bacterial cellular protein concentration as per Desvaux et al. [35]. A calibration curve was generated by converting bacterial cellular protein to cell mass concentration using bacteria grown on glucose. The protein concentration was measured using a commercial bicinchoninic acid assay (Sigma–Aldrich, St. Louis, MO, USA) at 565 nm using bovine serum albumin as a protein standard. All experiments were carried out in triplicate, and the results are presented as the average and standard deviations.

2.3. Culture Conditions and PHA Production

Minimal medium [3] was used for PHA production. In the case of agarose, dehydrated agarose was separately autoclaved before addition to media. The medium pH was adjusted to 7.5 before autoclaving. For inoculum preparation, 30 mL of sterile minimal medium using glucose as a carbon source in 250 mL Erlenmeyer flask was incubated on a rotary shaker for 24 h at 30 °C. The seed culture (30 mL) was transferred to 300 mL of sterile minimal medium in a 500 mL capacity flask and incubated at 30 °C in a shaking incubator.

The production of PHA was assayed by two approaches. In the first approach, the batch growth kinetics and PHA synthesis ability of *Sde* 2-40, *B. cereus*, and the co-culture were examined after being grown on xylan or agarose as the sole carbon source in minimal media. In the second approach, the biosynthesis of PHA in *Sde*2-40, *B. cereus*, and the co-culture was investigated by two-step batch culture under nitrogen source (NH_4Cl and yeast extract) limitation using agarose and xylan as the sole source of carbon. Inoculum (30 mL) was added to sterilized medium (300 mL) in a 500 mL capacity flask. First, cells were left in balanced nutrient environments for 100 h at 30 °C. After recovering biomass aseptically by centrifugation and washing with 2.3% (*w*/*v*) sterile NaCl solution, cells were added to sterilized medium (300 mL) without NH_4Cl and yeast extract as nitrogen sources in a 500 mL flask and incubated for 48 h at 30 °C.

2.4. Confocal Microscopy Studies

The bacterial cells were investigated for the presence of PHA accumulation by the staining method according to Ostle and Holt [36]. Briefly, 1% Nile Blue A (Sigma N0766, St. Louis, MO, USA) was prepared and filtered. Bacterial cell smears were prepared after heat-fixing and stained for 10 minutes. The excess stain was removed by water and washed with 8% acetic acid for 1 min. The smear was dried, remoistened with distilled water, and then observed at resolution of 200 nm under confocal laser scanning microscope (Zeiss LSM 710, Jena, Germany).

2.5. Analytical Methods

To quantify the PHA contents of the cells, culture samples were centrifuged, washed, and lyophilized. PHA was determined using gas chromatography (GC 6890N, Agilent Inc., Santa Clara, CA, USA, HP-5 column, 30 m × 320 μm × 0.25 μm). To the lyophilized cells, 2 mL of methanol acidified with H_2SO_4 (3% *v*/*v*) and 2 mL of chloroform were added and the mixture was heated at 100 °C for 3.5 h. After cooling, 1 mL of H_2O was added and the suspension was shaken for 10 min. The bottom organic phase was used for analysis. The detailed GC operating conditions are shown in a previous report [34].

2.6. Fourier Transform-Infrared Spectroscopy (FT-IR)

The collected, centrifuged, and lyophilized microbial cultures were added to potassium bromide (KBr) and pellets were prepared by using dry cells of *Sde* 2-40, *B. cereus*, and the co-culture of lyophilized cells at a ratio of 1:100 of cell sample:KBr, as suggested by Valappil et al. [37]. Absorbance spectra were recorded between 4000 and 400 cm^{-1} wave number values using a Nicolet model Magna-IR 200 FT-IR spectrometer. The diagnostic signals for PHAs (peak at 1728–1742 cm^{-1}) were taken as per Kansiz et al. [38].

2.7. Nuclear Magnetic Resonance (NMR) and Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

^{1}H NMR analysis of purified PHA was done using Bruker Avance-II 500 MHz spectrometer (Billerica, MA, USA) in $CDCl_3$ as a solvent. The monomeric composition of the polymer was determined by GC–MS, using an Agilent gas chromatograph coupled to an Agilent 5973 Mass spectrometer (Santa Clara, CA, USA). The chromatographic conditions used were: injection volume of sample = 1 µL, carrier gas (helium) flow rate = 1 mL/min, temperature of injection port = 230 °C, temperature of detection port = 275 °C, temperature ramp = 80 °C for 1 min, incrementing by 8 °C/min, and 220 °C for 12 min.

3. Results and Discussion

3.1. Growth of Sde 2-40, B. cereus, and Co-Culture on Agarose and Xylan

Growth of *Sde* 2-40 and *B. cereus*, and the co-culture of both strains were investigated. The maximum amount of protein biomass for *Sde* 2-40, *B. cereus*, and the co-culture using agarose (2% *w*/*v*) as a carbon source in minimal medium at 120 h was found to be 0.892, 0.0417, and 1.11 g/L, respectively (Figure 2A). The lag phase on agarose was slightly longer for *Sde* 2-40 as compared to the co-culture. The degradation activity of *Sde* 2-40 in co-culture increased as compared to the pure culture. To confirm this observation visually, a loop full culture from co-culture grown in flask was streaked on sea salt minimal media agar plate. The growth of *B. cereus* appeared after overnight incubation but *Sde* 2-40 was not visible until 20 h of incubation at 30 °C. However, *Sde* 2-40 colonies appeared on agar plate after 20 h of incubation and rapid increase in agar degradation on plates could be observed as the time progressed (Figure 2B). *Sde* 2-40 grown alone produced eumelanin and started to die after three to four days of incubation, as observed in earlier reports [3,34]. However, the colony growth and pit size due to agar degradation by *Sde* 2-40 increased in the presence of *B. cereus* and eumelanin was not produced even after ten days of incubation. This indicated that *Sde* 2-40 cells show active growth in the presence of *B. cereus* (Figure 2B). Figure 2C shows the growth characteristic of *B. cereus* on minimal sea salt agar medium. The agar degradation by *Sde* 2-40 was found to be very low when grown alone, as the pit formed by the colony growth was small and it did not spread far into the surrounding agar medium (Figure 2D). Eumelanin production was also observed with the appearance of black colour in agarose containing minimal sea salt media broth for the pure *Sde* 2-40 culture but not for the co-culture after 120 h (Figure 2E). This indicated that *Sde* 2-40 cells do not die in the presence of *B. cereus*, but instead grow well. Similar trends for *Sde* 2-40 growth were observed with xylan as the carbon source. The maximum amount of protein biomass for the co-culture from xylan was 1.00 g/L, as compared to 0.237 and 0.781 g/L for *B. cereus* and *Sde* 2-40, respectively (Figure 3).

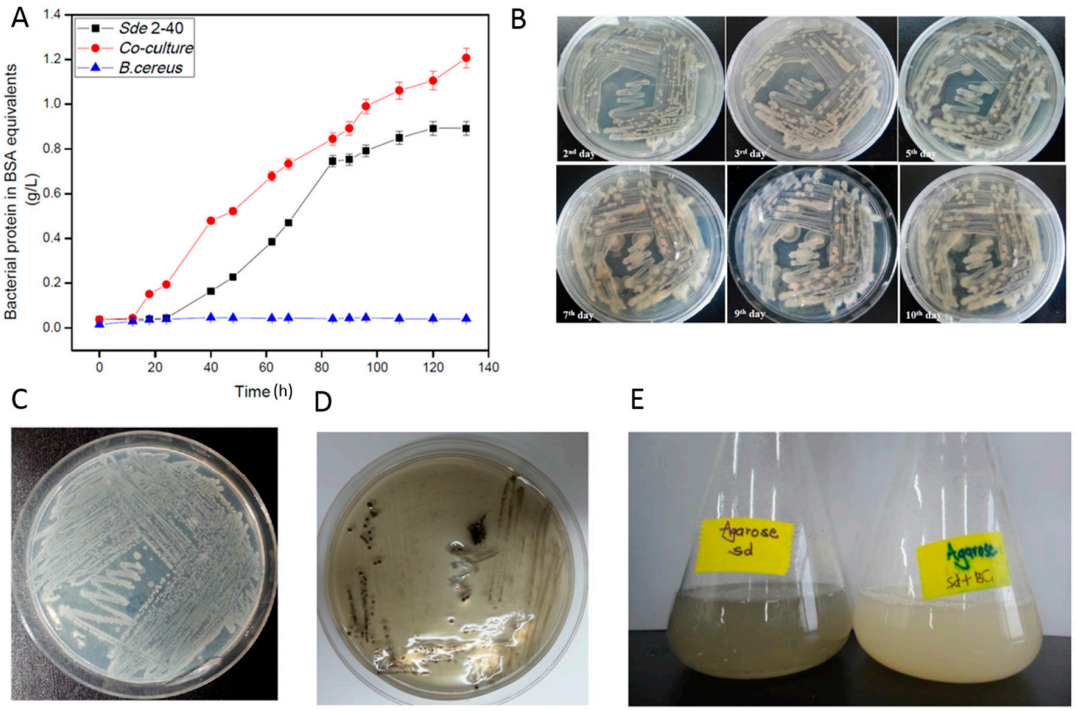

Figure 2. Characteristics of *Sde* 2-40 and *B. cereus*. (**A**) Growth profiles of *Sde* 2-40, *B. cereus*, and co-culture on 1% (*w*/*v*) agarose; (**B**) *Sde* 2-40 growing on sea salt minimal media agar plate along with *B. cereus*. Increase in pit size with increasing time is indicated by arrows; (**C**) Growth characteristics of *B. cereus* on minimal sea salt agar media; (**D**) Growth characteristics of *Sde* 2-40 where agar pitting colonies were turned black; (**E**) Pure culture of *Sde* 2-40 produced melanin while *Sde* 2-40 growing in co-culture with *B. cereus* did not produce melanin.

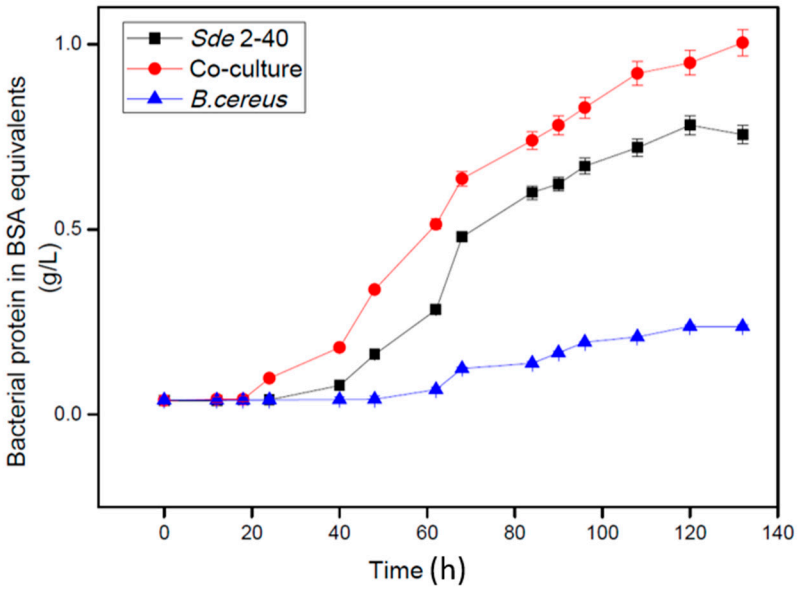

Figure 3. Growth profiles of *Sde* 2-40, *B. cereus*, and co-culture on 1% (*w*/*v*) xylan.

Sde 2-40 and *B. cereus* showed higher growth in co-culture. At this juncture, the exact nature of the interactions between *Sde* 2-40 and *B. cereus* are not known. Co-cultivation of various strains of lactic acid bacteria showed growth rate increase due to interchange of growth factors [24]. Provision of substrates for the enzymatic activity by a strain to another strain in co-culture can enhance growth

rate [39]. Growth-inhibiting products of one strain can be reduced by another strain and growth of co-culture companion can be improved [40]. Within or between different species, the reaction of microbes for existence of other cell types can be sensed [41]. Microorganisms in co-culture systems can interconnect through uninterrupted cell to cell interactions or via signal molecules in media [42]. The *Sde* 2-40 bacterium can produce proteins containing many cadherin and cadherin-like domains. These domains help in protein-protein interaction and bind directly to bacterial cell surfaces [43]. Cadherin and cadherin-like domains exhibit calcium dependent carbohydrate binding characteristics, which is very significant in resource-restricted aquatic environments [44]. Peterson et al. [45] described a commensal relationship between *B. cereus* and *Cytophaga-Flavobacterium* (CF) rhizosphere bacteria, where *B. cereus* peptidoglycan encourages the CF bacterial growth. Chen et al. [46] showed enhanced degradation of cypermethrin by co-culture of *Streptomyces aureus* and *B. cereus*. In their study, cypermethrin mineralization in minimal medium was low by either *B. cereus* or by *S. aureus*, but their combination consortium completely degraded cypermethrin. The authors correlated synergism with the partial and complete cypermethrin degradation abilities of *S. aureus* and *B. cereus*, respectively. Enhanced degradation of cypermethrin was credited to the capability of strain *S. aureus* to remove 3-phenoxybenzaldehyde [46]. Halverson and Handelsman [47] showed enhancement of soybean nodulation by *B. cereus.* The authors suggested that *B. cereus* seed treatments increased the acetylene reduction activity of soybeans by 25% to 73% compared with that of the untreated control. Dutta et al. [48] described that root colonization and promotion of growth can be affected due to root exudates prompting alterations in the cell surface of *B. cereus*. Dead microbial structures can possibly work as nutrient source for the growth of strains in the co-culture [49].

Sde 2-40 and *B. cereus* may have cell-to-cell interactions, which may help both to grow in the co-culture. The cadherin domains of *Sde* 2-40 and cell components of *B. cereus* may have active roles in the interactions. *B. cereus* may be providing some nutrients or metabolizing waste products generated in the media, thereby keeping *Sde* 2-40 in healthy and actively growing condition. This is an interesting observation which suggests that co-culture can be an innovative approach to increase the degradation rate of complex polysaccharides by *Sde* 2-40. These observations also indicate that the media which are being used commonly at present for culturing *Sde* 2-40 need proper optimization. The mechanism behind the enhancement of growth of *Sde* 2-40 by *B. cereus* needs further detailed investigation.

3.2. Confocal Microscopy Studies

Selective staining of PHA granules is commonly carried out by dyes Nile Red and Nile Blue A [36,50,51]. The bacterial cells cultured for 100 h at 30 °C were used to prepare heat-fixed smears. The cells containing PHA granules showed orange-coloured fluorescence after Nile Blue A staining (Figure 4(A1,B1,C1,D1)). The accompanying differential interference contrast (DIC) images in black and white show these granules within the cells (Figure 4(A2,B2,C2,D2)). The merged images of stained cells and DIC images are exhibited in Figure 4(A3,B3,C3,D3). In comparison to cell membrane or lipid-containing cell component, Nile Blue A demonstrates higher affinity towards PHA showing fluorescence [36]. Fluorescence in *B. cereus* cells after staining with Nile Blue A was not observed in agarose containing media, indicating the inability of *B. cereus* cells to produce PHA while growing in co-culture with *Sde* 2-40 on agarose (Figure 4(A1)). However, co-culture grown on xylan indicated fluorescence in *B. cereus* cells (Figure 4(B2)). This suggested that *B. cereus* also synthesized PHA along with *Sde* 2-40 in xylan containing media. PHA synthesized by *Sde* 2-40 while growing on agarose and xylan was clearly observed in Figure 4(C1,D1), respectively. Staining images also indicated a greater number of PHA containing cells in co-culture as compared to pure cultures. These results demonstrate that confocal microscopy can be an easy and inexpensive way to detect or differentiate the PHA accumulating cells in co-culture on the basis of cell morphology/phenotype.

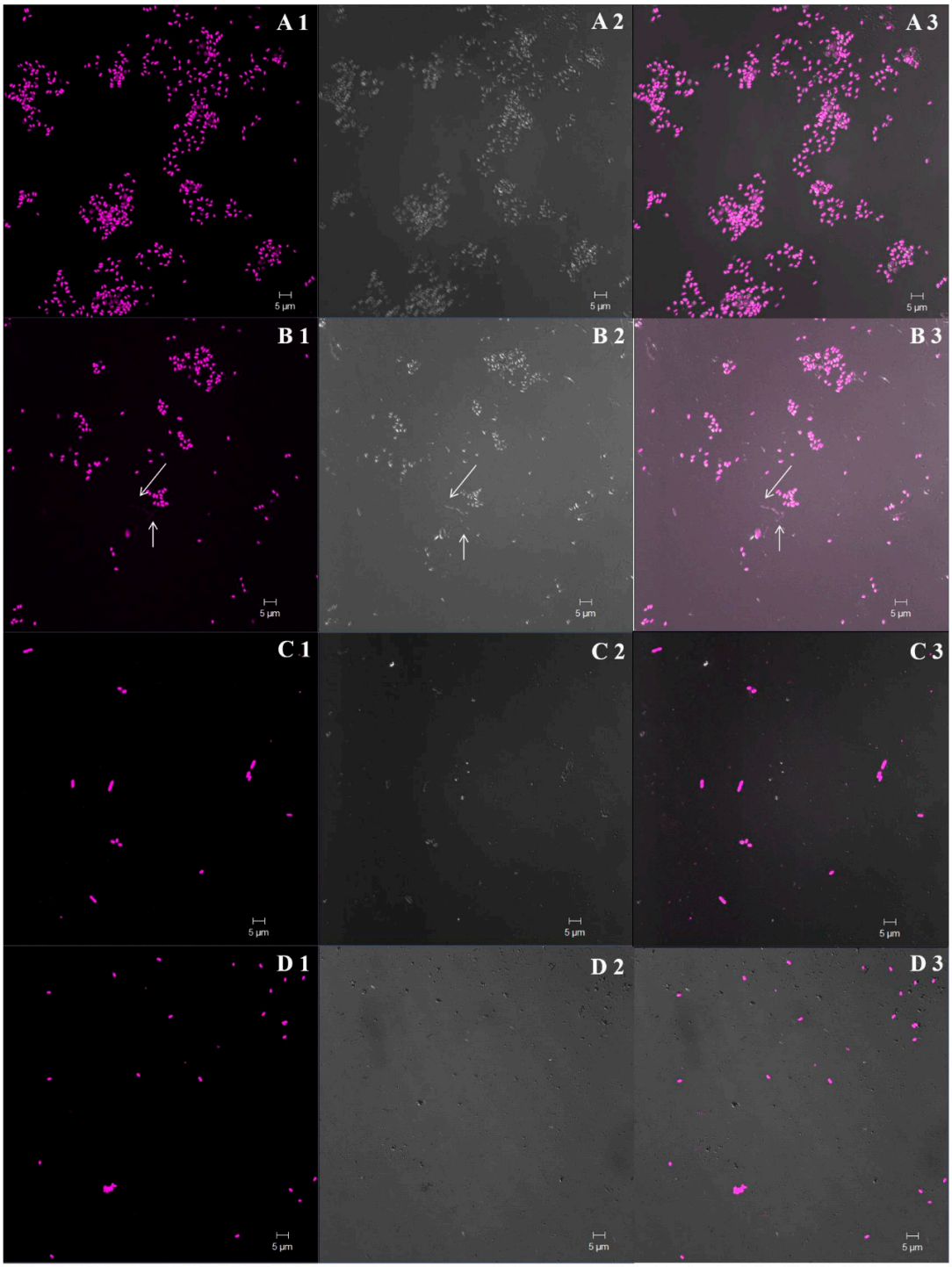

Figure 4. Confocal microscopy images of (**A**) co-culture grown on agarose; (**B**) co-culture on xylan; (**C**) *Sde* 2-40 on agarose; and (**D**) *Sde* 2-40 on xylan. The images in the panel indicate (**1**) stained cells; (**2**) differential interference contrast (DIC) image; and (**3**) merged image. Arrows indicate the *B. cereus* cells. *B. cereus* cells showing fluorescence are indicated by arrow in co-culture.

3.3. FT-IR Analysis

FT-IR method is useful for the rapid detection of cells containing PHA [52]. The ester carbonyl group stretching vibration (C=O) in PHA occurs at bands between 1724 to 1745 cm^{-1}. Bands at 1150–1300 cm^{-1} and 2800–3100 cm^{-1} correspond to C–O–C groups and C–H bonds, respectively.

FT-IR spectra on lyophilized bacteria in this study are illustrated in Figure 5. The characteristic PHB transmittance bands at 1724–1745 cm^{-1} and 1282 cm^{-1} were observed [52]. Bands at 1600 and 1700 cm^{-1} for amide I and band at 1544 cm^{-1} for amide II (polypeptides structures) of cellular proteins were detected, as reported earlier by Naumann et al. [53].

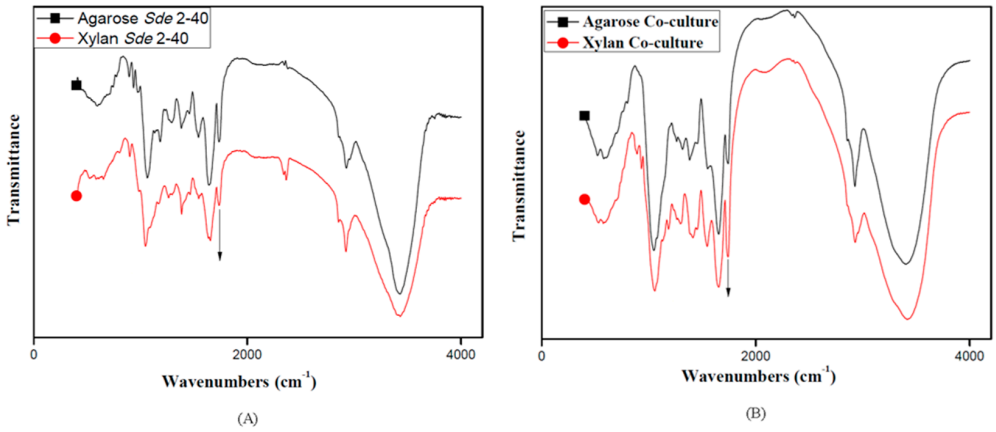

Figure 5. Fourier Transform-Infrared Spectroscopy (FT-IR) spectrum of PHA accumulating cells grown on different carbon sources. The characteristic PHA peaks are ester-carbonyl bond at 1727–1750 cm^{-1}. (**A**) *Sde* 2-40 and (**B**) co-culture.

3.4. NMR and GC–MS Analysis

The ^{1}H NMR spectrum given in Figure 6 shows three characteristic signal groups of PHB homopolymer: a signal at 1.2 ppm for methyl group coupled to a proton, doublet of quadruplet at 2.5 ppm for methylene group neighbouring asymmetric carbon atom with a proton, and multiplet at 5.2 ppm for methine group. The chloroform signal occurs at 7.2 ppm. According to Chen et al. [54], 3-hydroxybutyrate displays peaks at 5.2, 2.5, and 1.2 ppm for methine (CH), methylene (CH_2), and methyl (CH_3) groups, respectively. From this result, it can be concluded that the produced polymer is PHB. GC-MS data also show that the produced polymer by *Sde* 2-40 is PHB (Figure 7).

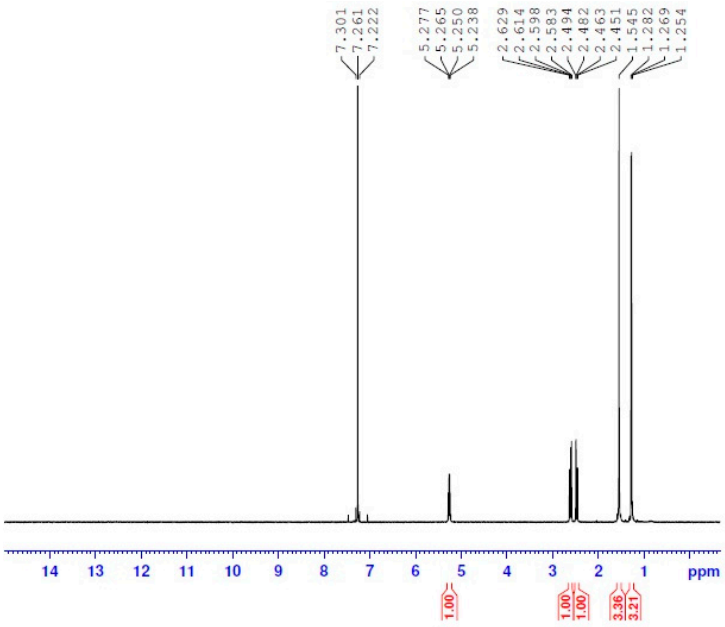

Figure 6. ^{1}H Nuclear Magnetic Resonance (NMR) spectrum of biopolymer obtained from co-culture of *Sde* 2-40 and *B. Cereus*.

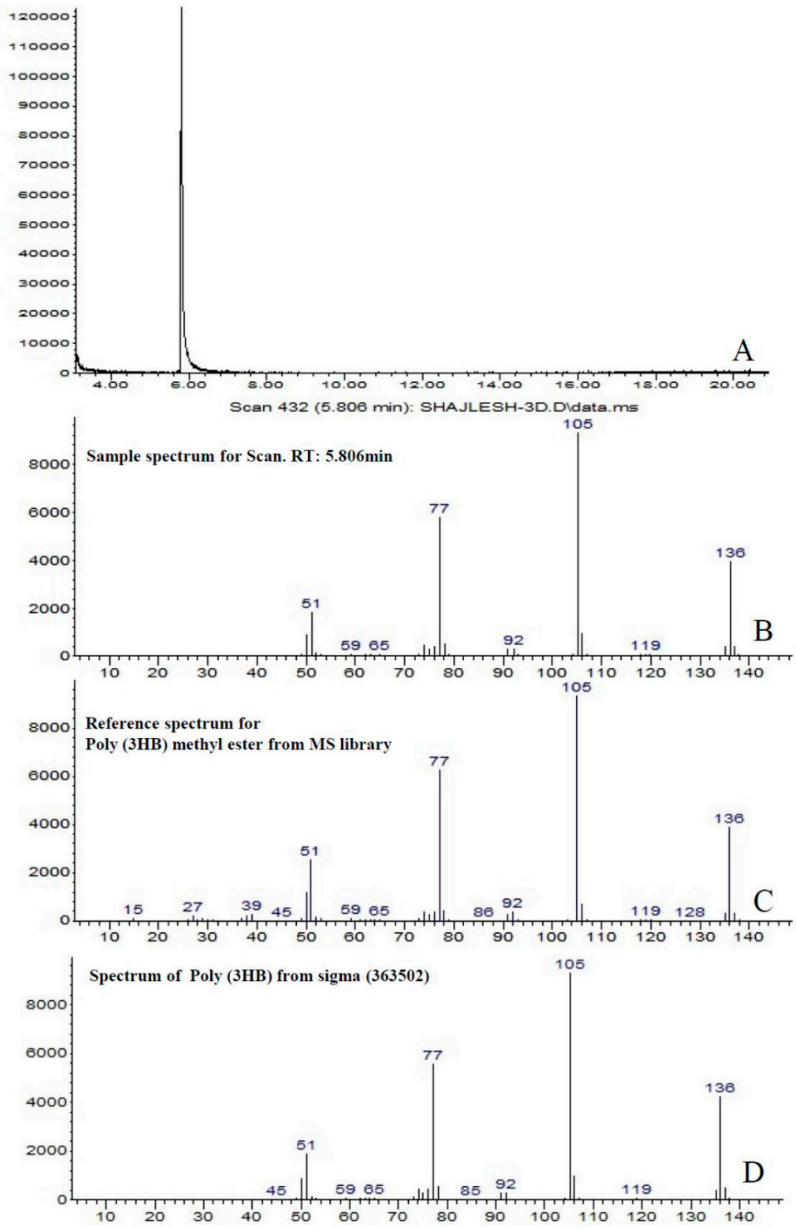

Figure 7. Gas Chromatography–Mass Spectrometry (GC–MS) analysis of biopolymer obtained from co-culture of *Sde* 2-40 and *B. cereus*. (**A**) Ion chromatogram; the peaks at 5.806 min are 3-hydroxybutyric acid methyl ester (3HB-Me); (**B**) Mass spectrum for the sample; (**C**) Reference mass spectrum for 3HB-Me from the MS library; (**D**) Mass spectrum of the commercial poly(3-hydroxyburyrate) (PHB) (Sigma 363502). The presence of the ions 51, 77, and 105 in the sample indicates PHB synthesis.

3.5. PHA Production from Xylan and Agarose

The ability of *Sde* 2-40, *B. cereus*, and co-culture of *Sde* 2-40 and *B. cereus* to produce PHA utilizing xylan and agarose was evaluated using two approaches (i) one step culture in nutrient balanced medium and (ii) two step culture (transfer of cells grown on nutrient balanced medium to nitrogen free medium).

The production of PHA using 2% (*w*/*v*) xylan and agarose as carbon sources was first attempted by growing the cells on nutrient balanced conditions in minimal media at 30 °C. The samples were collected for PHA analyses at 120 h. The PHA content from agarose for *Sde* 2-40 was 18.1% of dry cell weight (Figure 8A) and the PHA concentration was 0.240 g/L (Figure 8B). For co-culture,

the PHA content from agarose was 19.7% of dry cell weight and the PHA concentration was 0.295 g/L (Figure 8A,B).

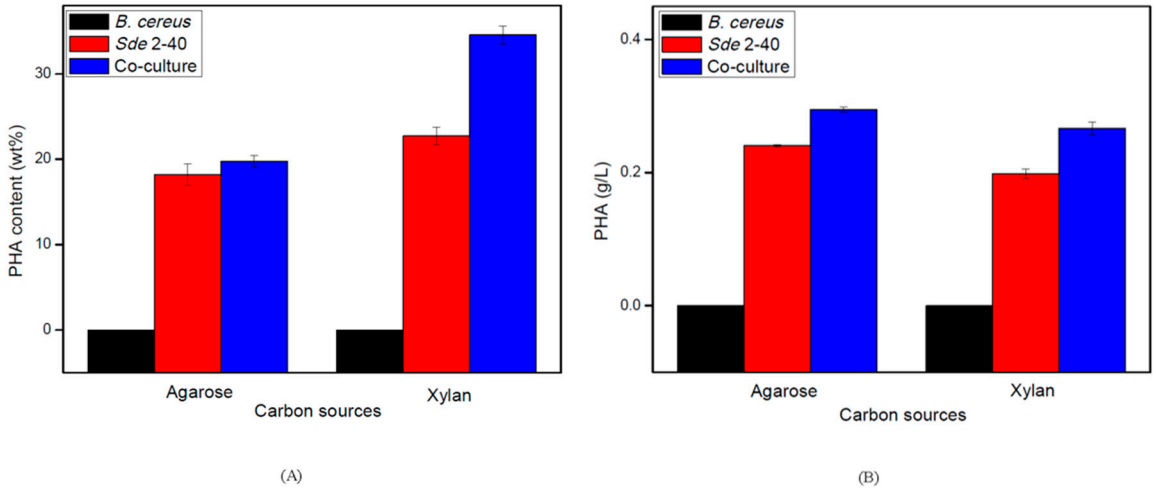

Figure 8. Production of PHA in one step culture in nutrient balanced medium. (**A**) PHA contents of *Sde* 2-40, *B. cereus*, and co-culture on agarose and xylan; (**B**) PHA concentrations of *Sde* 2-40, *B. cereus*, and co-culture on agarose and xylan.

Agarose is a neutral linear polysaccharide composed of alternating residues of 3-O-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose. It has been predicted that the agarolytic system of *Sde* 2-40 harbours agarose breakdown pathway consisting of five agarases [4,55]. The collective hydrolysis of agarose to simplest sugar units such as 3,6-anhydro-L-galactose and D-galactose happens due to these enzymes [55,56]. A study conducted by Shin et al. [6] using 0.2% agarose demonstrated the production of high levels of 3,6-anhydrogalactose by *Sde* 2-40. Therefore, we set a control experiment to test the efficiency of *B. cereus* to utilize galactose, which revealed that *B. cereus* can grow well on galactose containing media and produce low amounts of PHA (5.3% of dry cell weight) (Figure 9). This suggests that most of the galactose produced in the medium might be used by *Sde* 2-40 to produce PHA. This may be one of the possible and strong reasons for low PHA content from agarose by co-culture.

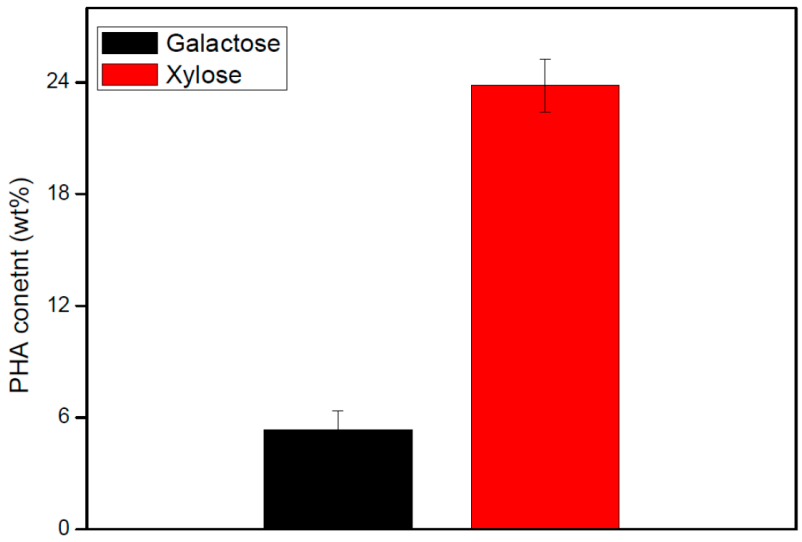

Figure 9. PHA production by *B. cereus* from galactose and xylose.

The PHA content from xylan at 120 h by *Sde* 2-40 was 22.7% of dry cell weight (Figure 8A), and the PHA concentration was 0.198 g/L (Figure 8B). In co-culture, the PHA content at 120 h was 34.5% of dry cell weight (Figure 8A), and the PHA concentration was 0.267 g/L (Figure 8B). *Sde* 2-40 has the ability to use xylans as a carbon source for growth and energy [1]. *Sde* 2-40 has a complete system for the degradation of xylan down to monomeric sugars [5,57,58]. The high amount of PHA production from xylan in co-culture made us to think that monomeric sugars may be produced extracellularly by *Sde* 2-40, which might be serving as carbon source for PHA production by *B. cereus*. If this is correct, *B. cereus* may have the ability to grow on xylose, a monomeric sugar produced by *Sde* 2-40 using xylan. Therefore, a control experiment was set in order to check the ability of *B. cereus* to grow and produce PHA using xylose as a carbon source. To our surprise, *B. cereus* displayed good ability to grow and produce 23.8% PHA of dry cell weight from xylose (Figure 9). This suggests that the released sugars in broth may probably be utilized by *B. cereus* in co-culture, thereby resulting in high content of PHA from xylan.

The second sets of experiments were performed to investigate the effect of nitrogen limitation on cells and PHA production. Reports of different researchers suggest that bacteria produce more PHA under the stress of nutrient limitation [59,60]. Glycogen, polyesters, and polyphosphates are accumulated as energy and carbon reserves [61]. These compounds are useful for cell survival in the case of famine. The first approach of one step culture in nutrient balanced medium for PHA production showed better results than the strategy of two step cell transfer. The PHA content in the cell transfer strategy from agarose was 18.4% and 15.3% of dry cell weight for *Sde* 2-40 and co-culture, respectively (Figure 10A). PHA concentration was 0.315 and 0.231 g/L for *Sde* 2-40 and co-culture, respectively (Figure 10B). Similarly, PHA content from xylan was 15.3% and 30.2% of dry cell weight (Figure 10A) and PHA concentration was 0.241 and 0.330 g/L for *Sde* 2-40 and co-culture, respectively (Figure 10B).

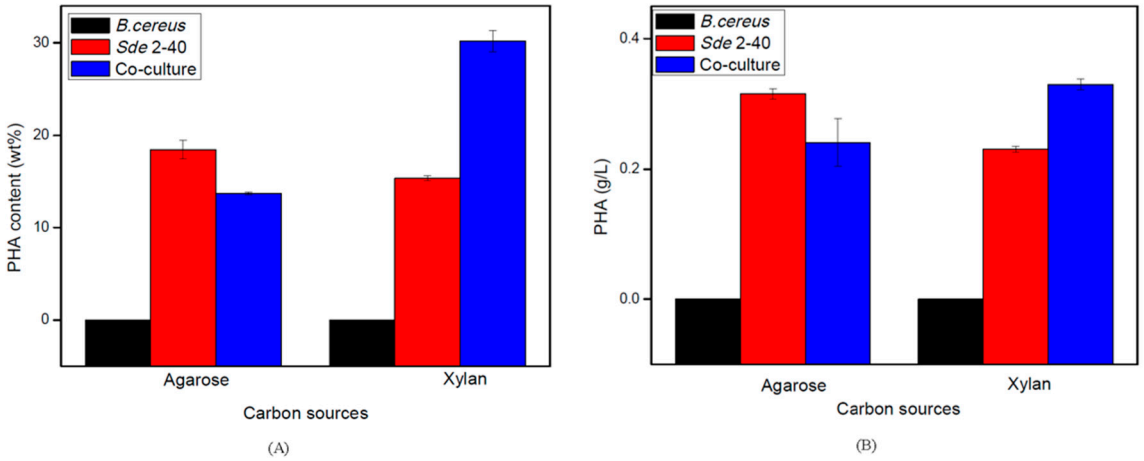

Figure 10. Production of PHA in two step culture. Cells grown on nutrient balanced medium were transferred to nitrogen free medium. (**A**) PHA contents of *Sde* 2-40, *B. cereus*, and co-culture on agarose and xylan; (**B**) PHA concentrations of *Sde* 2-40, *B. cereus*, and co-culture on agarose and xylan.

Marine bacterium *Sde* 2-40 is well known for the production of PHA using glucose [13] and starch [14]. The production of PHA by *Sde* 2-40 was reported to start at exponential growth phase and PHA accumulation of 0.58 g/L at 20 h and 0.53 g/L at 24 h were obtained using 20 g/L glucose and starch in marine broth, respectively [14]. The PHA production exponentially increased after carbon source feeding until fed-batch end and reached 21.3% of dry cell weight (2.71 g/L) using glucose and 17.4% of dry cell weight (2.04 g/L) using starch in marine broth during the fermentation period of 49 h [13]. Alva Munoz and Riley [12] reported PHA production by *Sde* 2-40 up to 1.5 g/L from 4% glucose in marine sea salt minimal media. However, the current study demonstrates the ability of *Sde* 2-40 to synthesize PHA from agarose and xylan for the first time. The PHA contents from agarose

and xylan were 18.1% and 34.5% of dry cell weight in one step culture and 13.6% and 30.2% of dry cell weight in two step culture, respectively.

Depending on the type of strain and conditions for bacterial culture used, PHA accumulation has been shown to be in the range of 1 to over 80% of dry cell weight [62]. Recombinant *Escherichia coli* was reported to produce 1.1% PHA in nutrient-rich microbial Lennox Broth which was increased to 37% after supplementation of arabinose or xylose in the xylan-based media [63]. In the present study, wild strains of the bacteria were used. The present study is the first report to show the highest production of PHA from xylan without pre-treatment by a wild strain of bacteria, to the best of our understanding. Although growth on 0.2% agarose of *Sde* 2-40 is reported [4,6], utilization of higher amounts of agarose in media by *Sde* 2-40 and simultaneous production of PHA has not been attempted yet. This is also the first report of PHA production from agarose. The PHA production by co-culture of *Sde* 2-40 and *B. cereus* from xylan in this study was higher (34.5% of dry cell weight) than the earlier report [63]. The results of this study are promising for reducing the production costs of large scale PHA production by utilising abundantly available cheaper carbon sources.

4. Conclusions

This study reports PHA production using wild type of bacteria, *Sde* 2-40 alone or in co-culture with *B. cereus*, grown using xylan and agarose as the sole carbon sources. Higher accumulation of PHA was observed in xylan grown co-culture. Confocal microscopic studies by Nile Blue A staining revealed PHA accumulating cells. The type of PHA produced was indicated to be PHB by GC–MS, FT-IR, and ^{1}H NMR analyses. This study demonstrated the production of PHA from inexpensive precursors without the need of hydrolysis or approaches of fibre reduction.

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