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PRODUCTION AND CHARACTERIZATION OF POLYHYDROXYALKANOATES (PHAS) PRODUCED FROM *BACILLUS CEREUS* MUL-A ISOLATED FROM A BIOGAS DIGESTER

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Abstract

Polyhydroxyalkanoic acids (PHA) are biodegradable polymers synthesized by microorganisms which can serve as alternative to conventional petrochemical based plastics. In the present study, twenty one bacterial isolates from one hundred & twenty six samples taken from anaerobic digester were screened for the PHA accumulation ability using Sudan Black B dye and further subjected to submerged fermentation to estimate the PHA production by maximum PHAs yielding strain. By 16S rRNA gene sequencing, the maximum PHAs yielding strain was identified as *Bacillus cereus* MUL-A. Effect of different physicochemical parameters such as temperature, pH, incubation time and composition of the modified PHA production media (PPMG) were studied on the polyhydroxyalkanoates (PHAs) yield. After 72 hours of submerged fermentation, a significantly high yield of PHAs i.e. 64.3% w/w were produced by the *Bacillus cereus* MUL-A strain by using PPMG medium having glucose (10 g/L) as carbon source and peptone (2 g/L) as nitrogen source at 35 ºC, pH 7.0. Provision of agricultural residues i.e. crude molasses and acid treated molasses as carbon source (6% v/v) & peptone (2 g/L) as nitrogen source in the PPMG medium has resulted in 36.9% and 44.6% biosynthesis of PHA content, respectively. Characterization of the purified PHA by Fourier transform infrared (FTIR) spectroscopy determined its purity and major functional groups.

Ke ywords: Bioproduct, Synthetic biology, Bioplastic, Bioconversion.

Introduction

Plastic materials that were originated from the petrochemical sources cause serious environmental threats due to their non-degradable nature. They are generally economical but their perseverance has a substantial negative environmental impact (Kehail & Brigham, 2018). Impending crisis of fossil fuel, alarming rate of petroleum prices and environmental impact associated with synthetic plastic lead the search for replacements in order to reduce the dependency of these non-renewable resources (Mohammed *et al.*, 2019). Therefore, biodegradable plastics produced by microorganisms serve as the best solution to this problem. They are eco-friendly in nature and are easy to degrade. There are different types of biodegradable plastics, commonly known as bioplastics, with different degrees of biodegradability. Among those, the polyhydroxybutyrate (PHBs) are the only one that is 100% biodegradable (Moreno *et al*., 2019).

Polyhydroxyalkanoates (PHAs) are the polyester of hydroxyalkanoates that accumulate in the microbial cells as reducing power or carbon/energy reserve material (Gamero *et al*., 2018). A wide variety of bacteria used to synthesize and store these inclusion bodies when grown under the stress conditions. These are intracellular granules without having any hazardous effects to the hosts (Abbondanzi *et al*., 2017). The physiochemical properties, structure, size, number of granules and monomeric composition may vary depending on the microbial communinty and carbon source used for growth (Kourmentza *et al.*, 2017). Among the bacterial species which accumulate large amounts of PHAs are *Ralstonia eutrophia* (Bozorg *et al*., 2015), *Azotobacter sp*. (Ryu *et al*., 2008), *Bacillus* sp. (Shah *et al*., 2016), *Burkholderia* sp. (Marang *et al.*, 2018), *Pseudomonas* sp. (Raza *et al*., 2016), *Halomonas* sp. (Bhattacharyya *et al*., 2012), *Haloferax* sp. (*Gao et al*., 2015), *Aeromonas* sp. (Koller *et al*., 2013), *Cyanobacteria* (Leong *et al*., 2017), *Chromobacterium* sp. (Srivastava & Tripathi, 2013) and recombinant *E. coli* (Koller *et al*., 2017) are most prominent.

The main limitations in PHAs production are the special growth condition required by microbe, cost of raw materials, fermentation processes, culture condition and significantly high recovery cost (Tai *et al.*, 2016). The cost of carbon source serves as about half (50%) of the cost for PHAs production. The agricultural and industrial wastes as well as their by-products i.e. waste water of paper mill, olive oil mill, sugarcane molasses etc. could serve as potential carbon source for PHAs production (Alsafadi & Al-Mashaqbe, 2017). Recycling of these waste materials for the production of polyhydroxyalkanoate is not only vital for management of agro-industrial waste but also in commercializing and economizing the bioplastic production (Westbrook *et al.*, 2019).

The present study was based on the determination of likelihood of molasses as a carbon source for microbial growth and PHAs production. Shake flask fermentation using different culture parameters were also optimized for maximum PHA accumulation.

Materials and Methods

Isolation and screening of PHA producing bacteria: One hundred & twenty six samples from the anaerobic digester of National Institute of Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan were collected for isolation of potential polyhydroxyalkanoates (PHAs) producing bacterial strains. Serially diluted (10^{-5}-10^{-6}) samples (100µl) were spread on the nutrient agar that were augmented with 1% glucose followed by overnight incubation at 35°C. For the screening of potential PHA producers, Sudan Black B (SBB) staining was employed.

PHA production and quantification: PHA positive bacterial isolates were subjected to submerged fermentation production for PHAs production followed by the quantification. For this, PHA production media (PPM) [composition: Na_2HPO_4 (6.78 $g^{-1}L$), $NaHCO_3$ (0.5 $g^{-1}L$), KH_2PO_4 (1.5 $g^{-1}L$), NaCl (0.5 $g^{-1}L$), $FeSO_4.7H_2O$ (0.02 $g^{-1}L$), 1.0M $MgSO_4$ (2 ml), 1.0M CaCl2 (100 µL) and trace element solution 1ml [composition: $ZnSO_4$ (0.1 $g^{-1}L$), H_3BO_3 (0.3 $g^{-1}L$), $CuSO_4$ (0.006 $g^{-1}L$), $NiCl_2.6H_2O$ (0.020 $g^{-1}L$), $Na_2MoO_4.2H_2O$ (0.030 $g^{-1}L$), $MnCl_2.2H_2O$ (0.25 $g^{-1}L$)] (Tsuge *et al*., 2018) was applied to screen out the best PHA producer bacterial isolate. The media was inoculated with 24 hour old 2% (v/v) bacterial culture and incubate in a shaking incubator for 24 hours.

PHA extraction: Sodium hypochlorite (NaOCl) digestion method was used to extract PHAs from fermentation broth (Lu *et al*., 2018). The bacterial cell biomass was collected by centrifugation of fermentation broth at 10,000 rpm for 20 minutes. After centrifugation, 1g of extracted biomass was taken, dissolved in 25 mL of NaOCl solution (0.4% v/v) and was incubated at 37ºC for 60 minutes. Later on, reaction mixture was again centrifuged for 20 minutes at 10,000 rpm in order to separate the PHAs pellet from rest of biomass. The pellet was first subjected to washing with acetone and then with distilled water. For purification of PHAs pellet, the extracted pellet was mixed with 10 mL of chloroform and allowed to stand overnight. The purified PHAs were weighed after the chloroform was evaporated. The PHAs yield was defined as the mass fraction of PHAs ($g^{-1}L$) in biomass ($g^{-1}L$) and calculated using formula given by Ghosh *et al*., 2019.

$$\text{PHA\%} = \frac{Weight\ of\ PHA}{Cell\ Dry\ Weight\ (CDW)} \times 100$$

Strain identification: Maximum PHAs yielding bacterial strains was identified using 16S rRNA gene sequencing technique By Macrogen, Inc, Seoul, Korea. The sequence analysis and alignments were carried out using NCBI BLAST tool. The nucleotide sequence under the accession number Bacillus cereus MUL-A (MN710592) was submitted to the NCBI depository. From GeneBank database, the sequence was compared and the related sequences were aligned using ClustalW. Using MEG5.0 program, the phylogenetic tree was constructed with 1000 bootstrap replicates by neighbor-joining method (Pichler *et al*., 2018).

Optimization of reaction conditions: Effect of physical and chemical factors including type of production media i.e. Liquid Broth (LB), PHA Production Media (PPM) and modified PHA Production Media (PPMG) [composition: PHA Production Media (PPM) + Glucose (5g/L)], temperature (25ºC, 30ºC, 35ºC and 40ºC), pH (6.0, 6.5, 7.0, 7.5 and 8.0) and time of incubation (24, 48, 72 and 96 hours) on the PHA production by *Bacillus cereus* MUL-A were studied. Furthermore, the effect of carbon source (fructose, glucose, sucrose and lactose) and nitrogen sources (tryptone, peptone, beef extract, meat extract and corn steep liquor) in the fermentation medium were studied to obtain maximum yield of PHAs (Mohandas *et al.*, 2017). The effect of agricultural residues as carbon source i.e. crude molasses (2, 4, 6 and 8% v/v) and acid treated molasses (2, 4, 6 and 8% v/v) having different concentrations in the fermentation medium were also studied for PHA production by *Bacillus cereus* MUL-A.

Fourier transform infrared (FTIR) analysis: The extracted polyhydroxyalkanoates (PHAs) were subjected to the Fourier transform infrared spectroscopy (FTIR) for its chemical structure analysis. For this, the biopolymer was dissolved in the chloroform solution and was then added to KBr pellets. The solvent was evaporated. Using a Perkin Elmer Fourier transform infrared (FTIR) spectrophotometer (Jasco FTIR- 6100, Japan), the infrared spectra of the samples were recorded and documented in the wave number range from 400 to 4000 cm^{-1} as reported by Sun *et al*., 2018.

Statistical analysis

All of the fermentation experiments carried out in this study were performed in triplicate. All the data with repeated measurements were compared statistically and was then evaluated by employing the analysis of variance (ANOVA). By using Student-Newman–Keuls test, the comparison between means were calculated and documented.

Results and Discussion

Among twenty one (21) strains of bacteria isolated from different samples taken from the anaerobic digester, ten (10) were found to be PHA producers as they show positive staining with Sudan Black B and were named as MUL-A to MUL-J (Table 1). Ali & Jamil, 2108 isolated bacterial strains from the soil samples taken from hilly areas of Muzafarabad and oil contaminated sites of Lahore Pakistan and were screened for their ability to produce PHAs using Nile blue A and Sudan black B staining methodologies. Similarly, Pattnaik *et al.,* 2018 used fourteen (14) chromium resistant bacteria were screened for their ability to produce PHAs by using indole acetic acid (IAA) and Sudan Black B method.

Table 1. Sudan black B staining results of bacterial isolates.

Sr. No.	Bacterial isolates	SBB staining	Sr. No.	Bacterial isolates	SBB staining
1.	MUL-A	+	12.	MUL-L	-
2.	MUL-B	+	13.	MUL-M	-
3.	MUL-C	+	14.	MUL-N	-
4.	MUL-D	+	15.	MUL-O	-
5.	MUL-E	+	16.	MUL-P	-
6.	MUL-F	+	17.	MUL-Q	-
7.	MUL-G	+	18.	MUL-R	-
8.	MUL-H	+	19.	MUL-S	-
9.	MUL-I	+	20.	MUL-T	-
10.	MUL-J	+	21.	MUL-U	-
11.	MUL-K	-			

+ = Positive; - = Negative

Bacterial isolates having PHA positive staining were employed to the submerged fermentation to study the relative PHA accumulation. After 24 hours of incubation, strain MUL-A produced maximum yield of PHAs i.e. 27.5% and was taken for further studies (Fig. 1). The high yielding PHAs strain MUL-A was identified as *Bacillus cereus* by 16S rRNA sequencing (Fig. 2).

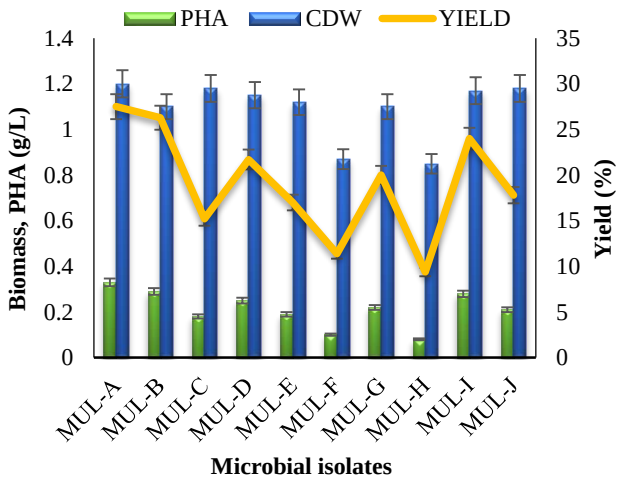

Fig. 1. Comparison of different microbial isolates for PHA production.

Optimization of different physical and chemical parameters is of prime importance in order to obtain high PHAs yield, particularly on the commercial scale. Three different types of media including liquid broth (LB), PHA production media (PPM) and modified PHA production media having glucose (PPMG) were screened for the production of PHAs. A significant high PHA yield i.e. 27.5% was obtained after 24 hours of incubation time using PPMG medium (Fig. 3). Kynadi & Suchithra, 2017 optimized fermentation broth for PHAs production using rubber seed oil as low cost substrate. PHA content of 604.77 mg/g dry cell weight (DCW) was obtained at an agitation speed of 150rpm after 72 hours of incubation using *Bacillus cereus*. By using response surface method (RSM), total PHA yield of 2.56 g/L was obtained with slight decrease in incubation time. *Bacillus cereus* FA11 produced maximum PHAs yield when a modified fermentation media i.e. glucose rich peptone deficient (GPRD) media is used (Masood *et al.*, 2017). Therefore, for further optimization studies, PPMG media was selected.

During temperature optimization, the PPMG medium was subjected to the incubation at different temperatures i.e. 25ºC - 40ºC. The optimum PHA yield was found to be 28.4% at 35ºC after 24 hours of incubation time (Fig. 4). As the incubation temperature was further increased from 35ºC, a drop in PHA yield was observed. This effect could be due to the enzyme degradation responsible for the PHA synthesis at higher temperature. De Grazia *et al.*, 2017 observed robust in PHA accumulation when the incubation temperature was raised gradually from 15ºC - 30ºC. It was also studied that the temperature alteration lowered the mass transfer efficiency and dissolved oxygen level which ultimately decrease in the synthesis of PHAs (Cho *et al.*, 2015). *Bacillus thuringiensis* SBC4 produced the maximum PHA content of 21.05% in the presence of corn cob as carbon source, 37ºC and after incubation time of 48 hours (Odeniyi & Adeola, 2017).

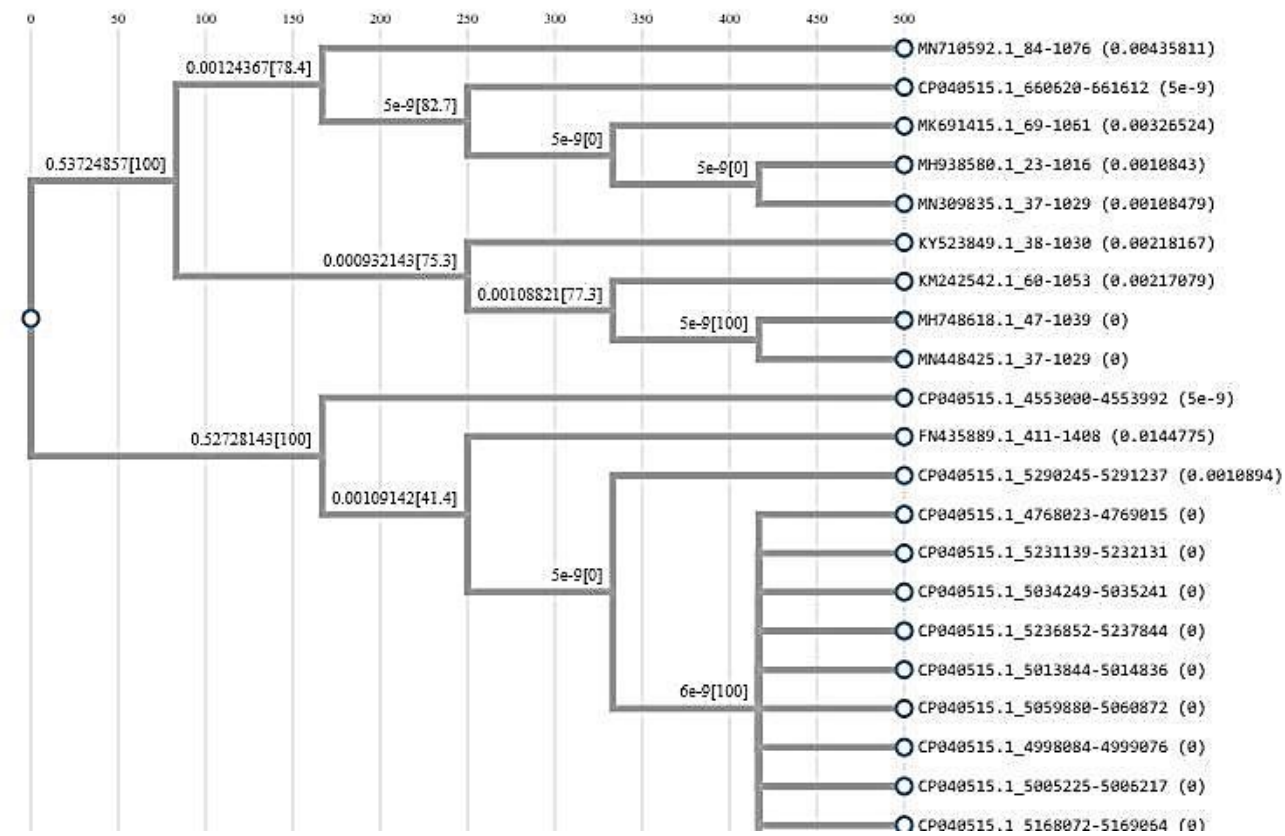

Fig. 2. Phylogenetic tree for the isolate *Bacillus cereus* MUL-A.

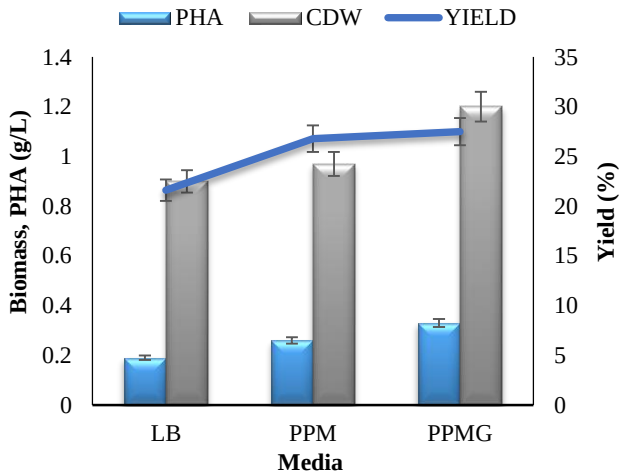

Fig. 3. Comparison of different media for PHA production by *Bacillus cereus* MUL-A.

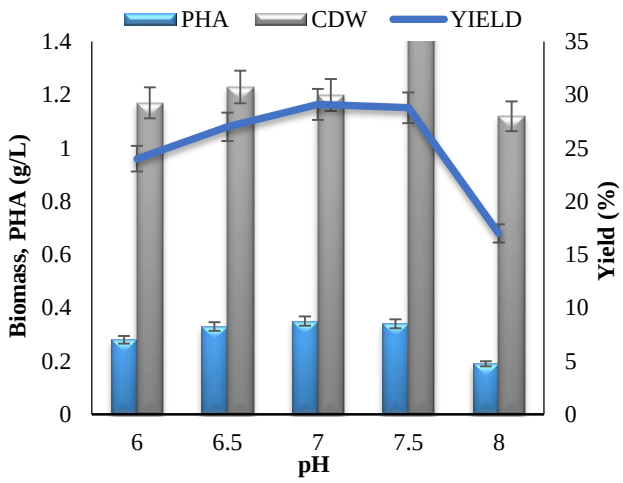

Fig. 5. Effect of initial pH on PHA production by *Bacillus cereus* MUL-A.

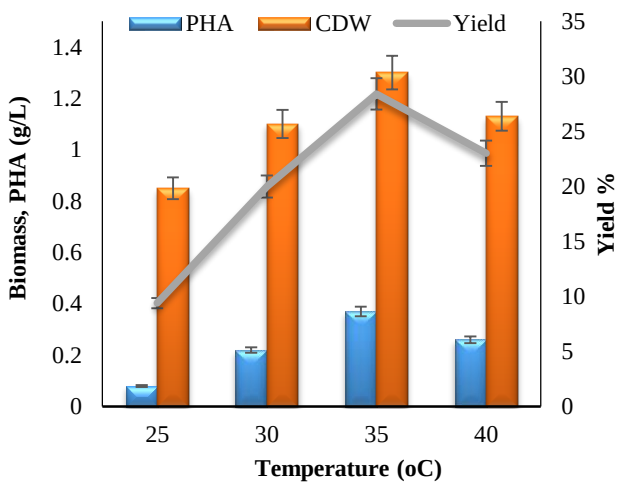

Fig. 4. Effect of temperature on PHA production by *Bacillus cereus* MUL-A.

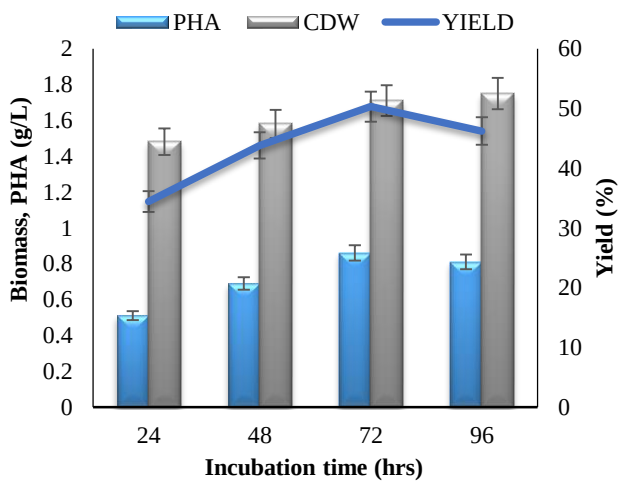

Fig. 6. Effect of incubation time on PHA production by *Bacillus cereus* MUL-A.

During the optimization of fermentation media, the influence of initial pH i.e. 6, 6.5, 7, 7.5 and 8 on the production of PHAs were studied. *Bacillus cereus* MUL-A gave a significantly high yield of PHAs i.e. 29.1% using the PPMG as production medium when incubated at 35ºC, at an initial pH of 7.0 and after 24 hours of incubation time (Fig. 5). The decreased final pH of the medium at the end of the fermentation process can ascribed to the entry of microbial cells from the exponential phase to the stationary phase. This behavior of PHA production is very much consistent with previous findings in which Madhumathi *et al*., 2016 reported that during un-buffered *Bacillus* submerged fermentation, low pH conditions prevent the degradation PHAs. Narayanan & Ramana, 2012 studied the similar effect of initial pH on the PHA production using *Bacillus mycoides* DFC1 strain and maximum yield was recorded at pH 7.3. During the controlled pH of 7.5, PHA content of 51% was achieved using mixed microbial culture using aerobic stirred tank bioreactor (Montiel-Jarillo *et al.*, 2017).

The production of PHAs were carried out by *Bacillus cereus* MUL-A for a period of 96 hours in order to determine the optimum harvesting time. A significant increase in the PHAs yield i.e. 50.3% was recorded after 72 hours of incubation time at 35ºC, pH 7 and at agitation speed of 150 rpm (Fig. 6). Increase in PHA content with increase in incubation time was observed until 72 hours after which gradual decline in overall PHA content was observed. Moreover, this reduction might be due number of factors including nutrient depletion, the decay of enzymatic systems that are responsible for the PHA biosynthesis, the consumption of PHA as carbon storage bodies by the cells under relatively more stress conditions as well as the inhibitory effect caused by high PHAs concentration (Flora *et al.*, 2010). Kumar *et al*., 2015 carried out studies of PHAs production by *B. thuringiensis* EGU45. Fermentation media supplemented with nutrient broth and 1% crude glycerol resulted in maximum production of PHAs after 48 hours of incubation. In a recent study a newly isolated strain of *Bacillus aryabhattai* T34-N4, delivered the 17% wt PHB of the cell dry weight when fermentation media is supplement with low cost starch (Bomrungnok *et al*., 2019).

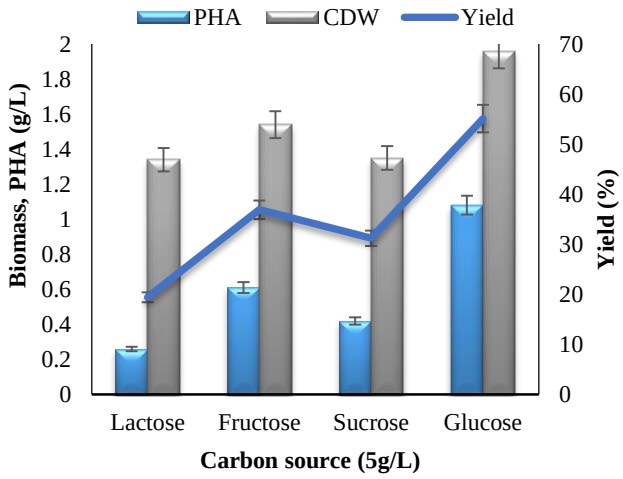

Fig. 7. Effect of different carbon sources on PHA production by *Bacillus cereus* MUL-A.

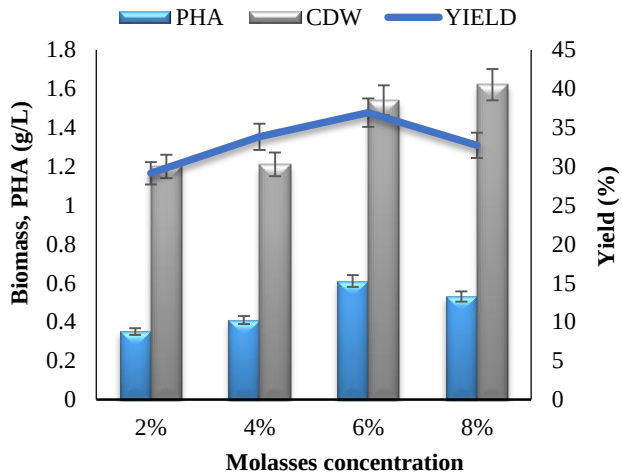

Fig. 9. Effect of different concentrations of crude molasses on PHA production by *Bacillus cereus* MUL-A.

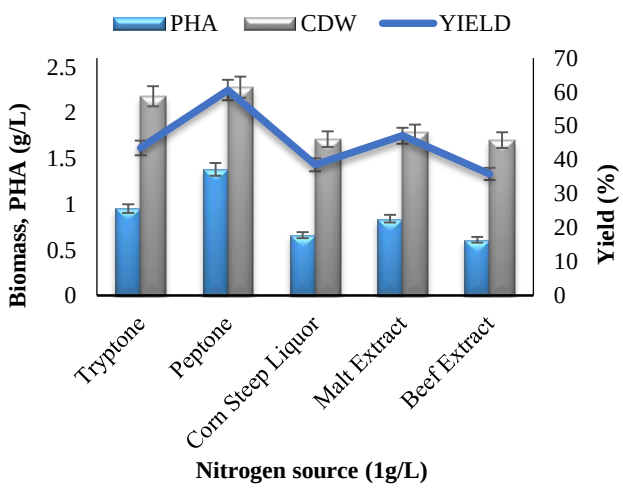

Fig. 8. Effect of different nitrogen sources on PHA production by *Bacillus cereus* MUL-A.

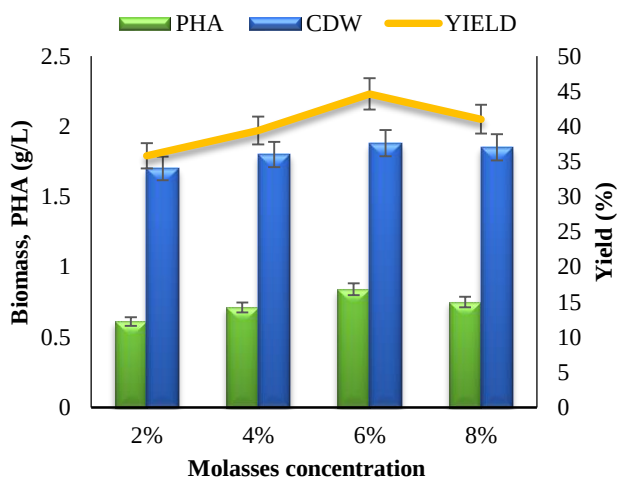

Fig. 10. Effect of different concentrations of acid treated molasses on PHA production by *Bacillus cereus* MUL-A.

In order to study the influence of various carbon sources on the yield of PHAs, synthetic carbon sources i.e., sucrose, lactose, fructose and glucose at concentrations of 5 g/L were supplemented to the PPMG medium. Compared with other carbon sources, significant increase in PHAs yield i.e. 55.1% was obtained by *Bacillus cereus* MUL-A when PPMG medium was supplemented with glucose (Fig. 7). Optimum concentration of various nitrogen sources, i.e. tryptone, peptone, corn steep liquor, malt extract and beef extract were evaluated by their supplementation to PPMG medium. The maximum PHAs yield of 60.5% was obtained by *Bacillus cereus* MUL-A strain in the presence of peptone in the PPMG medium after an incubation time of 72 hours, 35ºC, pH 7 and at agitation speed of 150 rpm (Fig. 8).

Agricultural residues i.e. crude molasses and acid treated molasses employed in the fermentation process as carbon source for PHA production by *Bacillus cereus* MUL-A. Being cheap and easily available carbon source, molasses serves as best agro-industrial waste for the production of PHAs. The provision of molasses can help to lower the overall cost of PHA production during the fermentation process. Molasses has very high contents of sugar i.e. 54%, w/w, comprising fructose (38%) and sucrose (62%) (Favaro *et al.*, 2019). It also contains vitamins such as pyridoxine, thiamine, niacinamide, riboflavin and trace elements (Acosta-Cárdenas *et al.*, 2018). In order to study the effect of crude molasses on PHA production, *Bacillus cereus* MUL-A was cultivated at initial concentration of molasses ranging from 2-8%. Crude molasses gave a maximum PHA content of 36.9% at concentration of 2% v/v in the PPMG medium at 35ºC, pH 7.0, incubation time of 72 hours and an agitation speed of 150rpm. The fermentation media was also supplemented with peptone as nitrogen source at concentration of 2 g/L (Fig. 9). Sanchez *et al.*, 2019 studied that there is a reduction in the concentration of both Cu and Zn elements by almost 25% when the mud had been removed from the crude molasses. When crude molasses was clarified using H_2SO_4 and was supplemented to the fermentation media as carbon source, an overall increase in the cell dry weight was observed with slight decrease in nitrogen contents (Anastopoulos *et al.*, 2017). Acid treated molasses gave maximum PHA yield of 44.6% at

concentration of 6% v/v by the *Bacillus cereus* MUL-A when grown in the PPMG medium at 35ºC, pH 7.0, incubation time of 72 hours and an agitation speed of 150rpm. The fermentation media was also supplemented with peptone as nitrogen source at concentration of 2 g/L (Fig. 10). Liberation of reducing sugar from crude molasses by the acid resulted in the higher yield of PHA (Luo *et al.,* 2018).

FTIR analysis of PHAs samples produced from *Bacillus cereus* MUL-A strain grown in the PPMG fermentation media supplemented with glucose was performed to identify the functional groups (Fig. 11). The absorption band around the 1392 cm^{-1} indicate the C-O stretching vibration. Moreover, absorption peaks at the wavenumber of 1447 and 1549 cm^{-1} can be ascribed to the C-H bending vibrations form –CH, $–CH_2$ and $–CH_3$ bonds. In the signature region of FTIR spectra a specific band observed near the wavenumber of 1647 cm^{-1} was linked to the C=O stretching vibration in the sample. An amorphous nature of polyhydroxyalkanoates (PHAs) was also associated with this band witnessed at 1647 cm^{-1}. Anti-symmetric stretching movement of CH_2 bond was observed at 2879 cm^{-1}. The absorption peaks observed in the region ranging from 3200–3300 cm^{-1} were consistent to asymmetric stretching of CH_3 bond (Porras *et al.,* 2017).

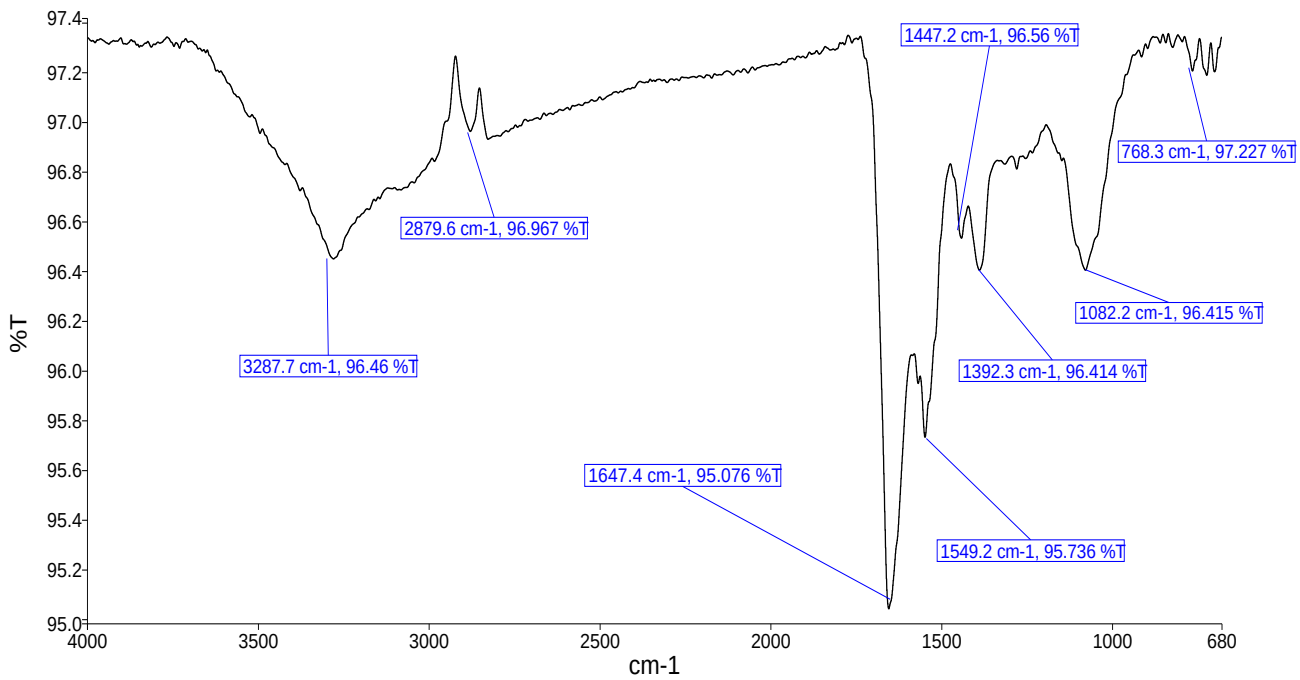

Fig. 11. Fourier transform infrared spectra analysis of PHA produced by *Bacillus cereus* MUL-A.

Conclusion

It is concluded from the present study that biogas digester are a potent source for the isolation of PHAs producing bacteria. It was observed that different cultural conditions have a significant effect on the PHAs production which in present study increased from 27.5% to 60.5%. Findings of the current study can be employed for designing a scale up process for PHAs production.

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