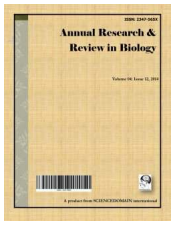

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Polyhydroxybutyrate Production by *Stenotrophomonas* and *Exiguobacterium* Using Renewable Carbon Source

Bushra Iqbal[1], Naima Khan[1] and Nazia Jamil[1*]

[1]Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan.

Authors' contributions

This work was carried out in collaboration between all authors. Author NJ designed the study, wrote the protocol and interpreted the data. Author BI anchored the field study, gathered the initial data and performed preliminary data analysis. While author NK managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The basic purpose of this study was to evaluate the potential of these bacterial strains to use arbon rich waste materials as nutrients and use them to produce cost effective polyhydroxybutyrate (PHB).
Study Design: Basic phenotypic and genotypic study of screening bacteria for low-cost PHB production.
Place and Duration of Study: Identified bacterial strains were taken from lab collection of Department of Microbiology and Molecular Genetics. This study was conducted between 2011-2012.
Methodology: Carbon rich waste materials as nutrients and use them to produce cost effective amount of PHB. Two indigenous bacterial strains *Stenotrophomonas* and *Exiguobacterium* were used in this study. The potato starch and waste water with minimum treatment were used as carbon sources and compared with glucose. The Fourier transform infrared spectroscopy was used

**Corresponding author: E-mail: jamil_nazi@yahoo.com;*

to confirm the presence of the polyhydroxybutyrate.
Results: The percentage PHB was 10.2%, 8.6%, 48.02% when glucose, potato starch and waste water was used as sole carbon source strain *Exiguobacterium respectively.* While for *Stenotrophomonas* the higher rate accumulation rate for PHB was 11.7%, 10.4% and 36.4% using glucose, potato starch and waste water as carbon source respectively. The Fourier transform infrared spectroscopy confirmed the presence of C=O in the PHB material extracted from the biomass. The genetic basis for the PHB production was also confirmed by sequencing p*haC* gene for the selected strains.
Conclusion: This study directs that environmental bacterial strains use many renewable carbon sources like waste materials to accumulate the PHB could be very cost effective. *Stenotrophomonas* and *Exiguabacterium* producing PHB using organic waste material nonetheless still there is a prerequisite to do more research on their production optimization and genetics.

Keywords: Polyhydroxybutyrate; Exiguobacterium; Stenotrophomonas; potato starch; waste water; Fourier transform infrared spectroscopy.

1. INTRODUCTION

Today there is a growing concern over the use of conventional plastics [1]. Production is reliant on depleting sources of hydrocarbons and waste that is produced from petroleum-derived plastics takes several decades to decompose, producing harmful toxic compounds during this process [2]. Bioplastic is defined as a form of plastic synthesized from renewable resources such as plant starch and microbial species. Many bacteria and archaea produce polyhydroxyalkanoates in restricted environments. Polyhydroxyalkanoates (PHAs) are bacterial storage polymers that accumulate when carbon levels are high but levels of another necessary nutrient, such as nitrogen, are low. These polymers are depolymerized and used by microbial species as nutrient sources.

The family of PHAs includes several polymeric esters such as poly(3-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), poly(hydroxybutyrate-co-hydroxyoctanoate) (PHBO). Poly(3-hydroxybutyrate) (PHB) is the most common natural microbial PHA. PHA synthases, the key enzymes catalyzing the polymerization of 3-hydroxyacyl-coenzyme A (CoA) into PHAs [3]. They use coenzyme A thioesters of hydroxyalkanoic acids (HA) as substrates and catalyze the polymerization of HAs into PHA with the concomitant release of CoA [4]. In some microbial species, accumulation of PHA occurs during the presence of excess carbon and a limitation of nitrogen sources [5]. The valuable characteristics of sludge, includes high energy and nutrient content, these charecteristics make sludge as a viable resource of energy instead of a waste [6]. However, bacteria can be grown on different media easily depending on their availability [7], so this study was conducted using renewable carbon sources for PHA production. The use of different waste materials for PHA biosynthesis is a good strategy as production is cost efficient, and disposal problems are also overcome [8]. Waste water is composed of a lot of substrates along with acetate and experiments were conducted using different compounds separately or in mixtures that includes propionate, butyrate, glucose, lactate but the metabolism of these organic substrates is not yet well understood [9]. The production of PHB from potato starch has been reported to emphasize the need to utilize the second most abundant starch source for the valuable biopolymers production [10].

2. MATERIALS AND METHODS

2.1 Screening, Growth Conditions Nile Blue and Sudan Black Staining

Two phylogenetically identified strains, *Stenotrophomonas rhizophila* and *Exiguobacterium profundum* with accession numbers KF270354 and KF928335 respectively obtained from MMG culture stock of Research Lab II, were used for this study. Strains were streaked on PDA agar (glucose 20 g/l, ammonium sulfate 2 g/L, potassium dihydrogen phosphate 13.3 g/L, magnesium sulfate 1.2 g/L, citric acid 1.7 g/L, trace elements 10 ml/L and agar 15 g/L) containing 5 µg/ml Nile Blue. Plates were incubated at 37℃ for 24-48 h. Plates were illuminated with UV light for detection of PHA producing strains. Heat fixed film of bacterial strains were prepared on a slide and slide was immersed in a filtered solution of Sudan black B (sudan black 3 g/L, ethyl alcohol 950 ml, distilled water 50 ml). Stained for 15 minutes. Drained

and air dried the slide. Immersed and withdraw the slide several times in Xylene and blot dry with absorbent paper. Counter stained for 5-10 seconds with 0.5% (wt/vol) aqueous safranin (safranin 5 g/L in water). Dried the slide and observed under 100X power lens of light microscope.

2.2 Analysis of Waste Water

Waste water was taken from the sewage of Kasur city, Pakistan. After collecting waste water, it was first autoclaved. Then it was centrifuged to remove larger particles. After that it was filtered. Carbohydrate and protein content was determined of waste water.

2.3 Pretreatment of Carbon Sources

Both the carbon sources were first pretreated. Potato extract was prepared by crushing and then lyophilized. Lyophilized powdered was mixed in water to prepare solution and autoclaved for sterilization. After that starch content was determined by comparing with the standard graph of starch. Waste water was taken from the sewage of Kasur. It was first autoclaved and then centrifuged and filtered. Carbohydrate and protein estimation were determined by H_2SO_4/Phenol method [11] and Bradford assay [12] respectively.

2.4 Time Profiling and for PHB Production

Medium PDA agar was prepared and autoclaved. Inoculums of pre-cultured respective strains were taken so that initial optical density was around 0.05 nm. The flasks were incubated at 37℃ and 150 rpm. After incubation 5 ml of the sample was collected and optical density was taken at 600 nm after 24, 48, 72 and 96 h. Biomass was calculated and finally PHB extraction was done by Sodium hypochlorite digestion method [10].

2.4.1 Extraction of PHB by sodium hypochlorite method

Cells were collected by centrifugation. Biomass was determined of dry pellet. In 8 g of biomass 100 ml of sodium hypochlorite was added and pellet was suspended by vortex [13]. Hundred ml of chloroform was added in it and incubated at 37℃ for 90 min on shaker. Centrifugation was done. Upper layer was removed and lower layer containing the chloroform was allowed to evaporate. The percentage of PHB was calculated.

PHB% was calculated as follows:

$$PHB\% = \frac{\text{Mass of PHB}}{\text{Mass of Biomass}} \times 100$$

2.4.2 Production of PHB from different carbon sources

Different (Glucose, Potato extract, waste water) carbon source was added in separate flask. Potato extract and waste water were used in the PHB medium in the initial stage by replacing glucose. Flasks containing 50 ml of PDA broth were prepared, supplemented with potato extract and waste water in respective labeled flasks. Two percentages of waste water, 100% waste water and 50% waste water were used. The bacterial strains were inoculated in the flasks and were incubated at 37℃ with 150 rpm. The growth was observed after 24, 48, 72 and 96 h of incubation.

2.5 *phaC* Gene Amplification

phaC gene amplification was done using degenerate primers [14] phal-1 (forward, 5′-CARACNTAYYTNGCNTGGMGNAARGA-3′) and phal-2 (reverse, 5′-TARTTRTTNACCCARTARTTCCADAT-3′). The polymerase chain reaction, reaction mixture (50 µl) using degenerate primers contained 15 µl of template DNA, 12.5 µl of master mix (provided by the manufacturer) 2.5 µl of each forward and reverse primer. The PCRs were run on 1% agarose gel and the DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

2.5.1 Amplified products purification

All PCR products were purified by PCR Purification Kit (Fermentas). All steps were done according to the instructions of the manufacturers. Isolated DNA was electrophoresed on 1% agarose gel in TBE buffer at 80 V. Staining of gel was done by ethidium bromide (0.5 µg/ml) solution.

2.5.2 *phaC* Gene sequencing

Amplified PCR purified products were submitted to Center of Excellence in Molecular Biology (CEMB) Lahore, Pakistan for sequencing. DNA sequencing was done by DNA sequencing system (Applied Biosystem, 3100/gas3100-1696-013), by Dideoxy DNA sequencing method.

3. RESULTS AND DISCUSSION

Stenotrophomonas and *Exiguobacterium* with accession numbers KF270354 and KF928335, were used. *Stenotrophomonas* is a Gram negative, non-lactose fermenting bacterium having and creamy in color while *Exiguobacterium* is a Gram positive, non-lactose fermenting bacterium and yellowish orange in color. Fluorescence was observed (Fig. 1) under UV. PHB production ability was further confirmed by Sudan Black staining by observing small black colored granules in the cells of both strains.

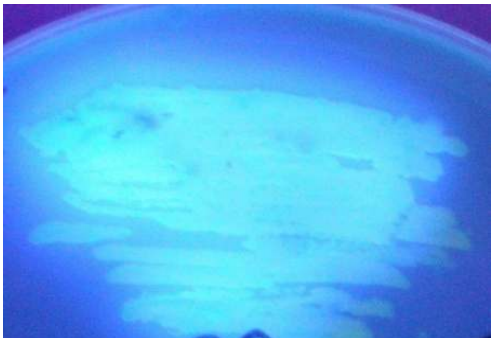

Fig 1. *Exiguobacterium* showing Fluorescence on Nile Blue plate. Fluorescence was observed under UV illuminator after grown on PDA plate containing 5 µl/ml Nile blue

Maximum biomass obtained from *Stenotrophomonas* was 31.2 g/l and PHB as 11.7% after 72 h when supplemented with glucose. When this strain was added with 2% potato extract the biomass was increased (52.1 g/L) as compare to PHB production (10.4%) (Table 1). In presence of 50% wastewater, growth trend appears not to associate with PHB production as it starts to decline after 72 h despite of increase in biomass. Maximum biomass was 18.6 g/L after 96 h and %PHB was 5.83% after 48 h. Growth trend was even different when 100% of wastewater was used. In this case, although the biomass was increasing (37.2 g/L). PHB production was maximum after 24 h (36.24%) Table 1. *Exiguobacterium* produced same amount of PHB with 50% waste water as *Stenotrophomonas* (Fig. 2b). This may be an indication that after 48 h, bacteria has started utilizing PHB because of depletion of glucose in the medium. Same trend in biomass and PHB production was seen for this strain when supplemented with 50% wastewater. Related observation was perceived for this strain when given with 100% wastewater. At start, PHB production was increasing with biomass till 24 h and later on PHB production start to decline. Carbohydrate and protein concentration (C:N) ratio was very important parameter for PHB production. Formation of PHB in *P. oleovorans* was induced by nutrient limitations. Highest levels were found when nitrogen was limiting, as also seen for PHB production in *Pseudomonas* sp. [15].

Both strains produced PHB in the presence of potato extract and waste water (Tables 1, 2) but production was less in these conditions [16]. The carbon sources used in this study are water soluble and easily separable from the biomass obtained. In this study potato starch and waste water has been used as carbon sources without any enzymatic or chemical treatment. These carbon sources have provided amounts of PHB comparable with the other commonly used carbon sources such as glucose and oils. Maximum biomass production was in case of potato extract but low PHB production because potato extract was a rich source of carbohydrates and it enhances the growth. PHB production was less in this case because when cell was growing in a large number did not accumulate PHB. Two concentrations of waste water were used. 100% waste water and a combination of 50% waste water and 50% salt solution were used. Biomass and PHB production was greater in case of 100% waste water may be because of high carbohydrate content as compare to 50% waste water. But both the biomass and PHB production were low as compare to glucose and potato extract.

The FTIR spectrum of extracted polymer sample was compared with that of polyhydroxybutyrate. The sample bands were observed similar with the standard spectra. The confirmation of carbonyl group signal at 1720 cm^{-1} by FTIR analysis proved presence of PHB [17]. Genotypic characterization by PCR based strategy was used after screening by Sudan Black staining to confirm PHB producers from different bacterial species [18,19]. In this study p*haC gene* fragment of 600 bp was amplified (Fig. 3). The amplified size of *phaC* gene not agrees with the size reported Kung et al. [14]. The nucleotide sequence of the PCR gene of *Stenotrophomonas* revealed homology with the sugar phosphate isomerases/epimerases gene of *Stenotrophomonas maltopilia* with a 16/16 (100%) identities and 0/16 (0%) gaps. The secretion of PHB from the cells bacteria uses type II secretion system (Figs. 4 and 5). For this purpose a protein responsible for the transport binds with the PHB and helps in its transport.

When *Exiguobacterium* amplified gene was showed sequence homology with the type II secretion system protein which helps in transport of PHB It can be concluded from present study that genetic diversity exit in reference to *polyhydroxybutyrate polymerase synthase C* gene and under study bacteria needs more molecular genetics work as these different in genetic arrangements as describe by Solaiman et al. [20] in *Pseudomonas* sp. While these Bacterial strains accumulate an enormous amount of polymers inside their cells; the real task is to use those carbon sources which can be easily separable from the biomass. The use of an inappropriate method and carbon source could contaminate the polymer extracted. The carbon sources used in this study are water soluble and easily separable from the biomass obtained.

Table 1. Time profiling of PHA production by *Stenotrophomonas* using different materials as carbon source

Time (hr)	*Stenotrophomonas*							
	Glucose (2%)		Waste water (100%)		Potato starch		Wastewater and Salt soln.	
	BM g/l	PHA %	BM g/l	PHA %	BM g/l	PHA %	BM g/l	PHA %
0	0	0	0	0	0	0	0	0
24	12.6	0.2	14.9	**36.24**	44.4	1.35	5.8	1.85
48	28.3	5.6	17.9	18.4	48.6	4.6	11.9	**5.83**
72	30.1	8.3	20.3	15.2	50.2	7.8	15.7	4.3
96	31.2	**11.7**	37.2	1.89	52.1	**10.4**	18.6	1.25

BM=Biomass of the bacterial strains

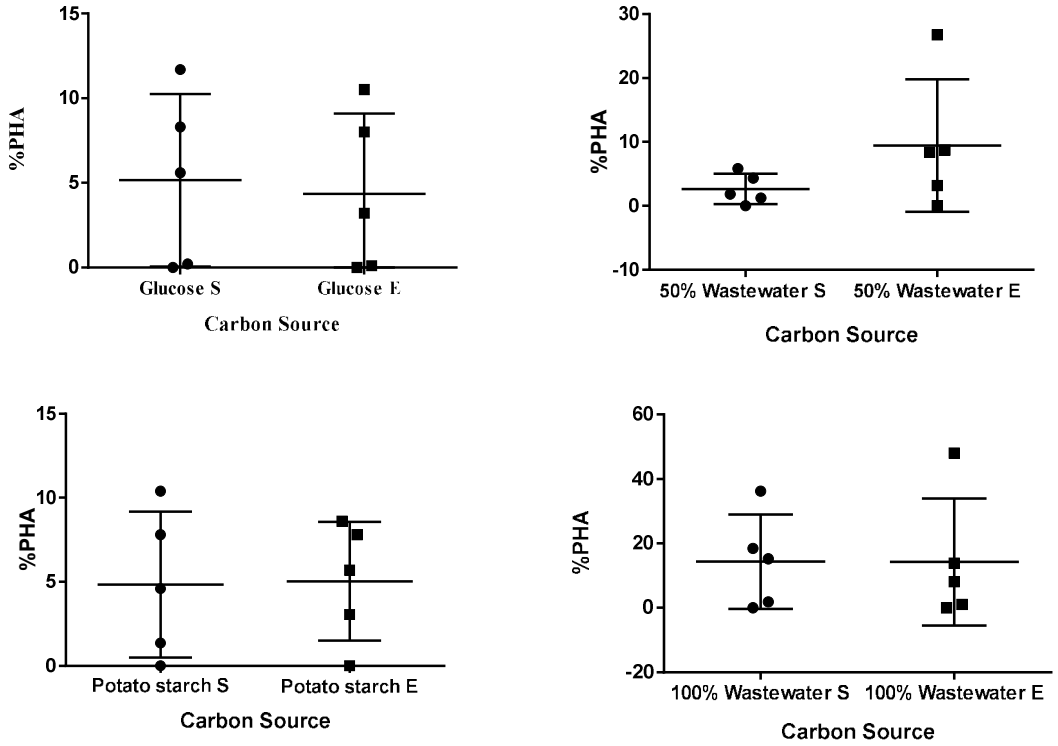

Fig 2. Comparison of % PHA production by *Stenotrophomonas* and *Exiguobacterium* using glucose, potato starch and 50 and 100% wastewater as sole carbon sources

E= Exiguobacterium, S= Stenotrophomonas

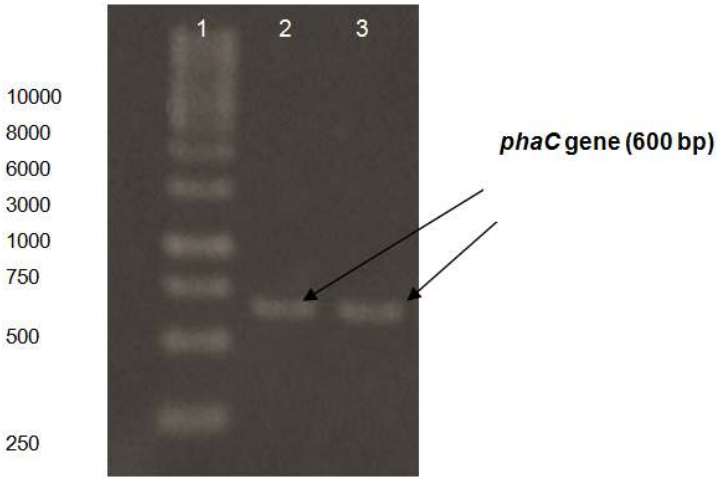

Fig. 3. Amplified products purification. Lane1: Ladder 1 kb DNA, Lane2: *Stenotrophomonas*, Lane3: *Exiguobacterium*

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A              CGCTGGCCGCGTGCTGGC--GCTGGCCAACACCC-CGCTGGGCTTCCGTCATGGGCCGAA
B              CGCGTTCAGCGCGACGGCCAGCCGGTTACCACCGATACCGTGCCGGTGTGGTGGGGTGGG
C              ------------------------------------CCTGGGATTTAGTCTTAGCGTCAG
                                                   * * *    **  * *

A              GTCGACGCTGGATGGCAACAC--CCTGGTGGTGGTGCTGCGCAGCGTGCAGCCGCTGGCG
B              GCAGGCACCGACGTGCAGCACAGTTTCTTCCAGGCGCTGCACCA-GGGCACCAGCATCAT
C              GCAG---CTGAGTGG-GACAC---TGGATTGTGGCCCCACACAATACTCCCTCGATACTG
               *  *   * *    *   ***       *   **  *  * *      *    *

A              CGCCGCTACGAACAGGACCTGCTGGAAGAACTGCGCCGCG-ACGGCGTGGCCGGCCAGGT
B              CCCGGCCGATTTCATCGGCTGCGTGCACAACGATGATCCGTACACGATCAATCACCAGGC
C              TAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTATGGCTCTCCGGTTCA--CACCA--T
                   *       *       *   * * * *       *   *    *      ***

A              GCTGGCGATCGGCCCGCATTCGGACATCG--GCGCCGAT-GACGAATACACCCTCA-CCG
B              ACTGCTGGCCAACCTGCTGGCGCAGACCGAGGCGCTGGCCAATGGCCAGGGCAGCG-ACG
C              GGTGCAGAACATCATCCATGAGAACTTGA-----CTCCTCAGTGCCAAGACCATCCTGTG
                 **  *  *  *   *    * *         *       *   *   *  *    *

A              TGCCGGCACTGGACGACCCGTGGCTGGCGCCGGTGTG-GCTGGGCTTCGCGCAGCTGTTC
B              ACCCGCATCGCGATTACCCG-GGCGGTCGCCCGAGCACGCTGATCCTGCTGGATGCGCTG
C              TACCTGCCAAGTATAGCCCT--TTGAGTGTTTTGGCC-ATCGAGCCTGATGGCTCAGTCG
                 **       *   ***         *     *     *  * *   *     *

A              GCGCTGCAGCGCTCGGCCGCGCTTGGCCTGACCC-CGGACAACCCGTTCCCGGATGGCAC
B              ACTCCGCAGGCGCTGGGCGCGCTGATCGCGATGTACGAACACGCGGTGTACGTGCAGT-C
C              CTTATAAAGAATATGAAGATATGATAGCCACTAAGTGTACCTGTCGTTAACAGAC--TCG
                      **    *                      * **    **   *

A              CGTCAACCGCGTCGTCAAGGGCGTC-ACCA-TCCACCAT--GGCTGA------------
B              GGTGATCTGGAACATCAATGCCTTCGACCAGTTTGGCGTCGAGCTGGGCAAGCAGCTGGC
C              TGTCAAGTAAAACCATGAGTGTCCTGAC-------------------------------
                ** *       *    *        **
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Fig. 4. Gene alignment of *Stenotrophomonas* amplified gene and isomerases gene of *Stenotrophomonas maltopilia.* (A) *Stenotrophomonas maltophilia* K279a. (B) *Stenotrophomonas maltophilia* R551-3 (C) Amplified fragment of *Stenotrophomonas* strain

Table 2. The time profiling of PHA production by *Exiguobacterium* using different materials as carbon source

Time {hr)	*Exiguobacterium*							
	Glucose		Waste water (100%)		Potato starch		Wastewater and Salt soln.	
	BM g/l	PHA %	BM g/l	PHA %	BM g/l	PHA %	BM g/l	PHA %
0	0	0	0	0	0	0	0	0
24	12.2	0.1	15.2	**48.02**	19.6	3.06	12.2	**26.75**
48	17.8	3.2	24.6	13.89	22.2	5.7	15.6	8.7
72	27.8	8	25.9	8.1	23.6	7.8	17.2	8.4
96	29.1	**10.5**	66.38	1.08	23.9	**8.6**	16.3	3.2

BM=Biomass of the bacterial strains

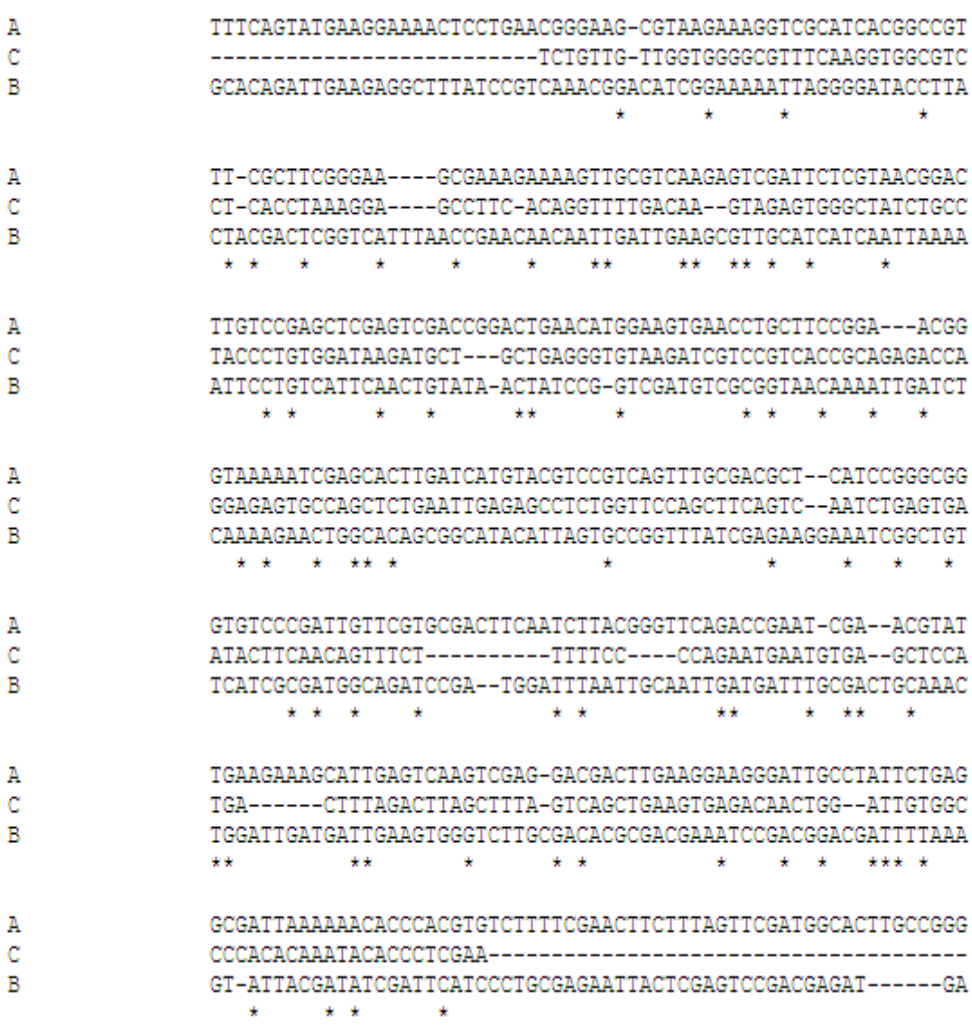

Fig. 5. Gene alignment of *Exiguobacterium* amplified gene and type II secretion system gene of *Exiguobacterium* strains. (A) *Exiguobacterium* sp. AT1b. (B) *Exiguobacterium sibiricum* 255-15 (C) Amplified fragment of *Exiguobacterium* strain

4. CONCLUSION

The carbon sources used in this study are cheaper, soluble and easily available for the production of PHB. Two strains (*stenotrophomonas* and *exiguobacterium*) were producing cost effective PHB. These strains can be exploited for large scale production. In this work two cheap carbon sources have been compared and in future these sources can combined to use for the production of polyhydroxybutyrates. However still there is a prerequisite to do more research on production optimization and genetics of these bacteria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rehm BH. Bacterial polymers: Biosynthesis, modifications and applications. Nat Rev Microbiol. 2010;8(8): 578-592.
2. Koller M. Poly (hydroxyalkanoates) for food packaging: Application and attempts towards implementation. Appl Food Biotechnol. 2014;1(1):3-15.
3. Lu Q, et al. Genetic and biochemical characterization of the poly (3-hydroxybutyrate-co-3-hydroxyvalerate) synthase in *Haloferax mediterranei.* J Bacteriol. 2008;190(12):4173-4180.
4. Rehm BHA, Steinbüchel A. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. Inter J Biol Macromol. 1999; 25(1–3):3-19.
5. Verlinden RA, et al. Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Aapp Microbio. 2007;102(6):1437-1449.
6. Tyagi VK, Lo S-L. Sludge: A waste or renewable source for energy and resources recovery? Renewable and Sustainable Energy Reviews. 2013;25: 708-728.
7. Jiang Y, et al. High poly (β-hydroxybutyrate) production by Pseudomonas fluorescens A2a5 from inexpensive substrates. Enz Microbial Technol. 2008;42(2):167-172.
8. Chaudhry WN, et al. Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. Ann Microbiol. 2011;61(3):623-629.
9. Munir S, Jamil N. Characterization of polyhydroxyalkanoates produced by contaminated soil bacteria using wastewater and glucose as carbon sources. Trop J Pharm Res. 2015;14(9): 1605-1611.
10. Ali I, Jamil N. Enhanced biosynthesis of poly (3-hydroxybutyrate) from potato starch by bacillus cereus strain 64-ins in a laboratory-scale fermenter. Prep Biochem Biotechnol. 2014;44(8):822-833.
11. Loewus FA. Improvement in anthrone method for determination of carbohydrates. Anal Chem. 1952;24(1):219-219.
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1):248-254.
13. Belal EB. Production of Poly-β-Hydroxybutyric Acid (PHB) by *Rhizobium elti and Pseudomonas stutzeri.* Cur. Res. J. Biol. Sci. 2013;5(6):273-284.
14. Kung SS, Chuang YC, Chen CH, Chien CC. Isolation of polyhydroxyalkanoates-producing bacteria using a combination of phenotypic and genotypic approach. Lett Appl Microbiol. 2007;44:364–371.
15. Annuar M, et al. Ammonium uptake and growth kinetics of *Pseudomonas putida* PGA1. Asia-Pac of Mol Biol Biotechnol. 2006;14(1):1-10.
16. Lu H, et al. Obtaining highly enriched cultures of Candidatus accumulibacter phosphates through alternating carbon sources. Water Res. 2006;40(20):3838-3848.
17. Shrivastav A, et al. Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates

(PHAs) production utilizing Jatropha biodiesel byproduct. Intern J Biol Macromol. 2010;47(2):283-287.

18. Sheu D-S, Lee C-Y. Altering the substrate specificity of polyhydroxyalkanoate synthase 1 derived from *Pseudomonas putida* GPo1 by localized semirandom mutage nesis. J Bacteriol. 2004;186(13): 4177-4184.

19. Solaiman DK, Ashby RD. Rapid genetic characterization of poly (hydroxyalkanoate) synthase and its applications. Biomacromol. 2005;6(2):532-537.

20. Solaiman DK, Ashby RD, Foglia TA. Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. Appl Microbiol Biotech. 2000;53(6):690-694.

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