ISSN 2709-3662 (Print)
ISSN 2709-3670 (Online)
*Journal of Agriculture and Food*
*2020, Volume 1, No.1, pp. 22-32*

# Optimization of Bioplastic Production by *Exiguobacterium sp.* and *Klebsiella sp.* Using Molasses as Carbon Source

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## Abstract

Bioplastics are the ecofriendly, biodegradable and biocompatible plastics originated from many living bodies including bacteria. Two bacterial strains *Exiguobacterium sp.* and *Klebsiella sp.* were screened for PHA production by Nile Blue Staining and Sudan Black B Staining. Both strains exhibited PHA production ability. Time profiling for the growth of two strains was performed using four different culture media. The two strains were optimized for PHA production for different pH conditions using three different carbon sources as glucose (control), sodium gluconate and molasses. Molasses sample was estimated containing 59.2% carbohydrate and 5.89% protein content by Phenol-Sulfuric Acid Method and Bradford Protein Assay respectively. Sodium Hypochlorite Method was used for PHA extraction. *Exiguobacterium sp.* showed exponential growth in molasses in 2% concentration and produced 54.0g/L biomass, 7.2 $gL^{-1}$ or 13.3% PHA at 78 hours. With molasses as carbon source, biomass production *by Klebsielliasp.*was recorded optimal with 2% supplementation producing 88.7g/L biomass and 8.5g/L or 9.58% PHA, after 78 hours of incubation at 37 ºC. Fourier Transform Infra-Red Spectroscopy analysis showed that *Exiguobacterium sp.* produced poly (3-hydroxybutyrate) in medium supplemented with glucose as well as molasses as confirmed by the presence of C = O group at 1624.712 cm-1 and 1385.783 cm-1 respectively.

**Keywords**: Exiguobacterium, Klebsiella, polyhydroxyalkanoates, molasses, FTIR
Article History: **Received:** 12th February; **Revised:** 5th May, 2020 **Accepted:** 5th May, 2020

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## Introduction

Increase in human population has led to the accumulation of large amount of non-degradable waste materials across our planet. The accumulation of plastic wastes has become a major concern in terms of the environment (Suriyamongkol et al., 2006). Conventional petroleum-based plastics pose many problems as they not only generate the recalcitrant wastes which take many decades to be decomposed in nature, but also generate toxins during the process of degradation (Akaraonye et al., 2010). For this reason, there is special interest in producing plastics from materials that can be readily eliminated from our biosphere in an eco-friendly fashion.

Polyhydroxyalkanoates (PHA) have been attracting considerable attention as biodegradable substitutes for conventional polymers (Lee and Choi., 1998). They have similarity of structural and chemical properties to those of conventional petrochemical plastics. Yet PHAs are biodegradable and can be produced from renewable resources (Thellen et al., 2008). Polyhydroxyalkanoates are the polymers of hydroxy alkanoic acids which accumulate as carbon reserve material in response to the ample supply of carbon when growth is limited owing to the starvation of other nutrients such as nitrogen phosphorus, magnesium and potassium (Hong et al., 2000) or oxygen and an excess amount of a carbon source is still present. While the most common limitation is nitrogen, for some bacteria, such as Azotobacter sp. the most effective limitation is oxygen. On the basis of number of the carbon atoms constituting the polymer, PHAs are classified into two distinct categories; short chain length (SCL) PHAs and medium chain length (MCL) PHAs. Moreover, another class of PHAs also exists comprising the polymers composed of SCL-MCLmonomers.

It is advantageous for bacteria to store excess nutrients inside their cells, especially when their general physiological fitness is not affected. PHA is synthesized by using three enzymes and their encoding genes (Steinbuchel and Hein, 2001; Reddy et al., 2003). The first enzyme β-ketothiolase encoded by phaA gene is responsible for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA; acetoacetyl-CoA reductase enzyme encoded by phaB gene involved in the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA (in case of PHB production) (Steinbuchel and Schlegel, 1991) and the PHA synthase, the product of phaC gene involved in the polymerization of (R)-3-hydroxybutyryl-CoA monomers (Rehm, 2003; Suriyamongkol, 2007).

Today, polymers have become a necessary part of contemporary life pertaining to their durability and resistance to degradation. Petroleum based synthetic polymers are found to be recalcitrant to microbial degradation. Therefore, serious efforts are mounted to the development of biopolymers with appropriate properties and processability, the ‘‘green’’ polymers (Tripathi et al., 2012). Present study indicates potentials of Exiguobacterium sp.

and Klebsiella sp. for production of polyhydroxyalkanoates using molasses and sodium gluconate.

## Materials and methods

***Selection and morphology of Bacterial Strains***

Two phylogenetically identified bacterial strains; Exiguobacterium sp. and Klebsiella sp. were obtained from MMG stock. The two bacterial strains were streaked on the surface of solid medium followed by incubation for 24 hours at 37ºC. After incubation, the plates were recovered from incubator and morphology of the isolated colonies was observed. Gram staining was done to check the cell morphology of the bacterial strains

***Screening for PHA Production***

For the detection of polyhydroxyalkanoates producing capability, the two bacterial strains were grown on solid PHA Detection Agar medium(Lee and Choi, 1999) (for 24 to 72 hours) at 37ºC to check the turbidity of colonies.The bacterial strains were also grown on solid PHA Detection Agar medium containing 25-50µl (per100ml of the medium) Nile blue dye (0.5 mg ml-1 in methanol), for 24 to 72 hours followed by UV illumination of the plates for green fluorescence.Sudan Black B staining was done for the detection of PHA granules inside the bacterial cells. PHA inclusions appear as blue-black droplets while the cytoplasmic parts of the cells appear pink.

***Pretreatment of Molasses Sample***

Cane molasses collected from a local sugar mill was used as the carbon source for the production of polyhydroxyalkanoates (along with glucose and sodium gluconate) after pretreatment. Two different pretreatment methods used; clarification and activated carbon treatment. The molasses sample was pretreated with a little modification of the method. The 5ml molasses sample was diluted up to 15oml (1:30) with distilled water and filtered through filter paper. The filtrate was centrifuged at 6000rpm for15 minutes for maximum clarification. The clear supernatant collected after repeated centrifugation was autoclaved and checked for carbohydrate content by Phenol-Sulfuric Acid Method. Three percent (w/v) activated carbon was added to the molasses, and after 24 hours incubation at 37ºC, insoluble parts were removed by centrifugation at 6000rpm for15 minutes. The supernatant was subjected to the estimation of carbohydrate and protein content. Phenol-Sulfuric Acid Method was used for estimation of carbohydrate content of Molasses (Sadasivam and Manickam, 1996). Estimation of Protein Content of Molasses Sample was done using by Bradford Protein Assay (Rosenberg, 1996).

***Bacterial Growth Optimization for PHA Production and extraction***

The two bacterial strains were grown in PHA detection broth using three carbon sources (glucose, sodium gluconate and molasses) at two different concentrations as 0.1% and 2%. The pH of the media was set at three different values as 5, 7 and 8. For each strain flasks containing 100ml PDA broth were prepared supplemented with one carbon source at a time. The labeled flasks were inoculated with 7% inoculum having been standardized to

0.5 optical density at 600nm. The flasks were then incubated at 37ºC for three to four days. Optical density, biomass and PHA percentage were recorded at 0, 8, 24, 32, 48, 5, and 78 hours of incubation.

Ten 10ml of each bacterial culture was taken and centrifuged in pre-weighed centrifuge tubes at 14,000 rpm for 5 to 10 minutes. The pellet dried overnight at room temperature and weighed. Eight grams of the pellet was treated with dispersions of 100ml sodium hypochlorite and chloroform each. The centrifuge tubes were then incubated at 30ºC in shaking incubator for 90 minutes and centrifuged at 14000 rpm for 5 to 10 minutes. After centrifugation three phases appeared in the tube. The bottom layer was that of chloroform containing the PHAs dissolved in it. This layer was pipette out with Pasteur pipette, dispensed in pre-weighed glass vials and allowed to dry. After the chloroform had evaporated the glass vials were weighed.

The percentage PHA was calculated as:

PHA (%) = [Weight of PHA/Weight of biomass] ×100

***FTIR Analysis of PHA***

PHA produced by Exiguobacterium sp. was analyzed by Fourier transform infra-red spectroscopy to identify its monomer composition. The selected strain was grown in media containing different carbon sources for 48 hours at 37ºC. After incubation, cell biomass was collected by centrifugation at 14000rpm for 5 to 10 minutes. The pellet obtained was dried overnight at 65ºC and treated with KBr in 1:5 ratio. This mixture was then used in FTIR analysis.

## Results

***Selection and morphology of the strain***

Exiguobacterium sp. and Klebsiella sp. were selected for PHA production. The colonies of Exiguobacterium sp. appeared as raised and light orange circularones with the entire margins where the colony size was about is 2mm.

Klebsiella sp. grew as 2-3mm sized off-white circular colonies with entire margins and convex surface. After Gram staining, the cells of Exiguobacterium sp. were seen microscopically as isolated Gram-positive cocci whereas those of Klebsiellasp. as Gram negative rods in chains.

***Screening for PHA Production***

When streaked on PHA agar medium, both of the strains showed off-white turbid growth on the agar surface. These strains also showed green fluorescence when grown on PDA medium with Nile blue dye. After Sudan Black staining, both strains showed typical blue-black granules of polyhydroxyalkanoates (PHAs) inside their cells while rest of the cells appeared pink under microscope.

***Pretreatment of Molasses Sample***

The clarification method was not suitable as it involved the dilution of sample and resulted in the reduced content of carbohydrate while the activated charcoal treatment was more suitable in clarifying the molasses sample as well as retaining its carbohydrate content (59.2%). For estimating the carbohydrate content of molasses sample, a standard curve was plotted between the optical densities of known standard samples taken along Y-axis and concentration of carbohydrates along X-axis. From standard curve, the molasses sample was found containing 592 µg ml-1 or 0.59g ml-1 or 59g 100 ml-1 carbohydrates. For estimating the protein content of molasses sample, a standard curve was plotted between the optical densities of Bovine Serum Albumin (BSA) standard samples of known concentration, taken along Y-axis and concentration of proteins along X-axis. From standard curve the molasses sample was found to contain total proteins as 58.93 µg ml-1 or 0.05893 g ml-1 or 5.893g 100ml-1.

***Time Profiling of Bacterial Strains in Different Growth Media***

The two bacterial strains were grown in four different growth media. Exiguobacterium sp. showed best growth in the medium containing molasses as carbon source where the strain showed exponential growth up to 78 hours after which the growth showed a decline. Klebsiella sp. also showed best growth in the medium containing molasses as carbon source up to 78 hours while with glucose the growth increased fairly high up to 33 hours. Growth medium comprising of molasses and water was also not supportive to growth.

***Optimization of PHA Production under Different Conditions***

By optimization, the optimum pH for both strains was found to be 7.0. In the control medium containing 2% glucose, Exiguobacterium sp. produced 1.97 g L-1 PHA (8.56%) from 23.0g/L biomass while the pH of the media was set at 7.0. The strain produced higher biomass of 23.6g/L in the medium containing 0.1% glucose but the PHA accumulation was lowered to 6.25% as compared to the medium containing 2% glucose.The same strain when grown in two different concentrations of molasses produced 7.20g/L PHA (13.3%) form 54.0 g L-1 biomass optimally in the medium containing 2% molasses as compare to 0.1% concentration of the carbon source where the same strain produced biomass up to 30.10g/L with a maximum PHA as 0.8 g L-1 initially at 24 hour of growth while the maximum percentage of PHA obtained was 8.3% (Figures 1– 3).

Exiguobacterium sp. showed good trend of growth and PHA production utilizing sodium gluconate as carbon source. The strain produced biomass up to 27.2g/L yielding 10.5% PHA when concentration of carbon source was 2%. However, with 0.1% sodium gluconate, biomass was greater as 36.22g/L with a yielding 3.03% PHA. After performing the optimization of Klebsiella sp. for different carbon sources used at two concentrations, the strain was found producing up to 23.5 g/L biomass and 10% PHA when the PDA

medium was supplemented with 2% glucose. However, with medium containing 0.1% glucose the same strain produced biomass up to 21g L-1 with a maximum PHA as 6.97%. Klebsiella sp. exhibited high capability of PHA production when grown in PDA broth supplemented with 2% molasses yielding 88.9g L-1 biomass with a maximum PHA as 9.58 %. PHAyield was, however, not fairly high in the medium containing 0.1% molasses as the same strain produced 9.6g L-1 biomass and 1.04% PHA. PDA broth supplemented with 2% sodium gluconate, Klebsiella sp. showed a reduced yield whereas with 0.1% concentration of sodium gluconate was found asoptimum as 33.5g/L biomass, 0.5g/L or 7.46% PHA at 56 hours (Figure 4 – 6).

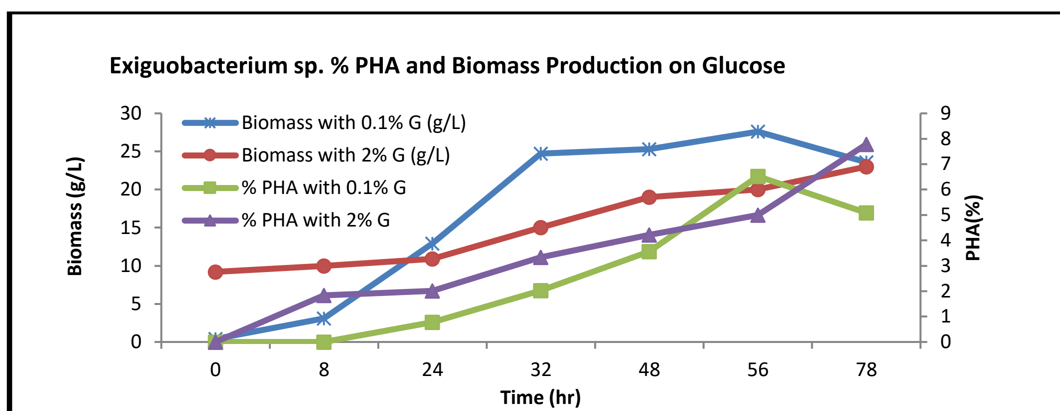


**Figure 1.** % PHA and Biomass production by Exiguobacterium sp. on Glucose

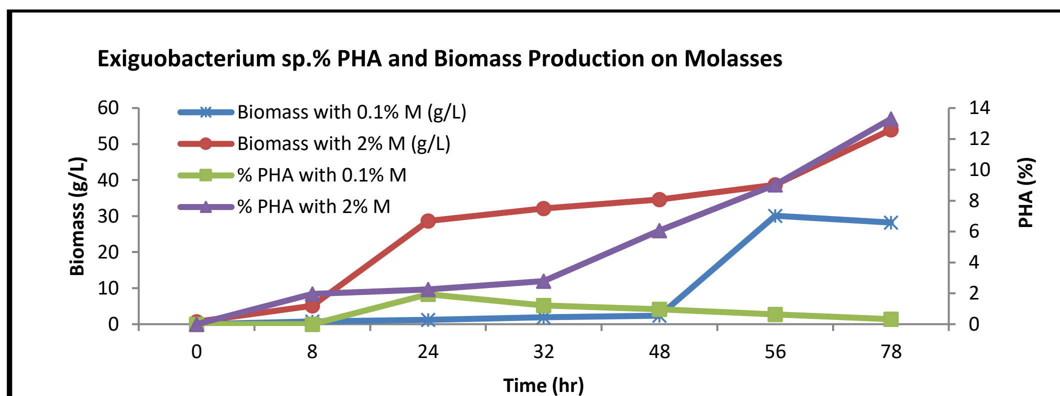


**Figure 2:** % PHA and Biomass Production by *Exiguobacterium* sp. on Molasses

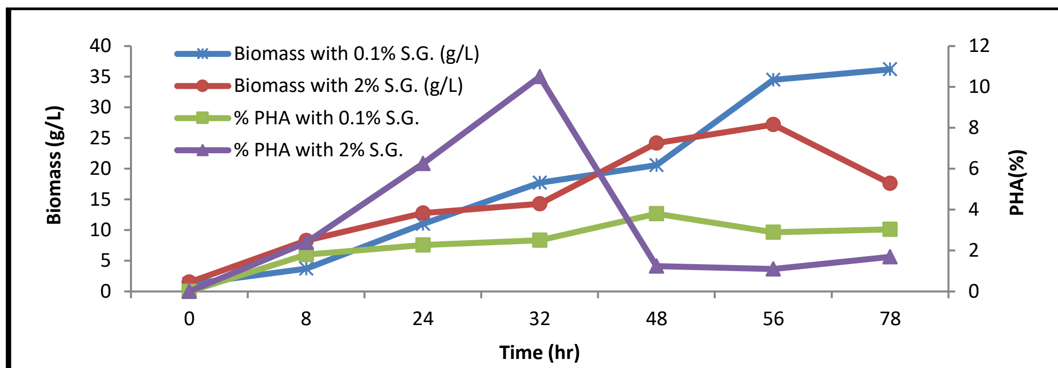


**Figure 3.** % PHA and Biomass Production by *Exiguobacterium* sp. on Sodium Gluconate

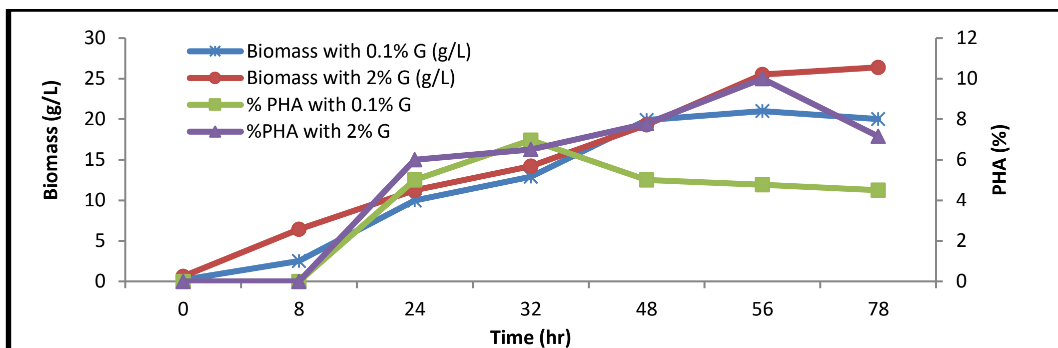


**Figure 4.** % PHA and Biomass Production by *Klebsiella* sp. with Glucose

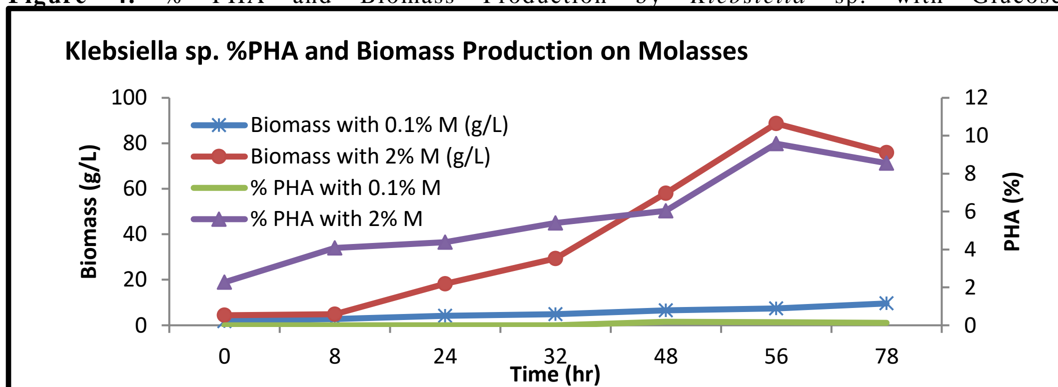


**Figure 5:** % PHA and biomass production by *Klebsiella* sp. with molasses

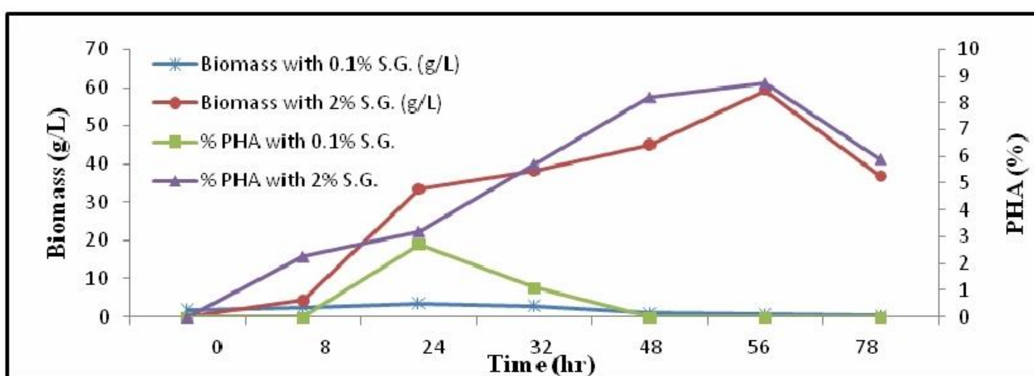


**Figure6.** %PHA Biomass Production by *Klebsiella* sp. with Sodium Gluconate as Carbon Source

***Fourier Transform Infrared Spectroscopy Analysis of PHA***

The FTIR spectrum of sample-KBr pellets of Exiguobacterium sp. was compared to that of PHAs in literature and found within the wavelength range given for PHAs in literature. Transform InfraRed Spectroscopy analysis showed that Exiguobacterium sp. produced poly (3-hydroxybutyrate) in medium supplemented with glucose as well as molasses as confirmed by the presence of C = O group at 1624.712 cm-1 and 1385.783 cm-1 respectively.

## Discussion

Polyhydroxyalkanoates (PHAs) have recently been the focus of attention as a biodegradable and biocompatible substitute for conventional non degradable plastics (Akaraonye et al., 2010, Sohail et al 2020). This research was aimed at screening the PHA producing bacterial strains and optimizing their PHA production trends under different conditions of carbon source and pH, analysis of its structural composition through FTIR spectroscopy was also carried out.Time profiling for growth of the two bacterial strains were also observed in different media. Exiguobacterium sp. showed the best growth in the media containing molasses as carbon source and exponential growth observed after78 hours and showed a stationary phase followed by decline. The growth was also fairly good in the medium consisted of PDA and glucose as it increased up to 54 hours. Water was found to be poor medium for growth. The growth was also very poor in the medium consisted of water and molasses. Similarly, Klebsiella sp. showed exponential growth in the medium containing molasses as carbon source up to 48 hour whereas in the rest of the three media growth increased up to 33 hours followed by a stationary and then decline phase. After wards PHA production was analyzed by both strains using glucose, sodium

gluconate and pretreated molasses as carbon source in the PHA detection broth. was proved as a good carbon source as Exiguobacterium sp. produced 13.3% PHA optimally in the medium containing 2% molasses as compare to the control medium containing 2% glucose where the same strain produced 8.56 % PHA while the pH of the media was set at 7.0.The results are comparable to those obtained by Tripathi et al., (2012) where increased biomass and PHA content observed in sugar refinery waste. The limitation in biotin content can act as an inducer for PHA production (Rehm, 2010).When grown in medium containing 0.1% carbon source at pH 5 Exiguobacterium sp. showed PHA maximally at 48 hoursin the medium with glucose as well as molasses. However, in the medium containing 0.1% sodium gluconate, PHA was detected after 48 hours of incubation. The same strain when grown in the medium with pH set at 7.0 showed good trend of PHA production. In control medium containing 0.1% glucose as carbon source PHA was found increasing consistently with biomass with the passage of time up to 56 to 48 hours with molasses followed by a decline in PHA percentage. With sodium gluconate at pH 7.0, the bacterium showed a different trend such as PHA was high initially, then decreased and after 24 hours increased again up to 48 hours while the biomass was increasing consistently with the passage of time. At pH 8.0 Exiguobacterium sp. produced PHA increasing with biomass up to 48 hours but with 0.1% molasses and sodium gluconate biomass increased up to 24 hours but PHA showed a different trend. The bacterium was however found producing 13.3% PHA optimally in the medium containing 2% molasses, as compare to the control medium containing glucose 8.56% PHA was recorded while the pH of the media was set at 7.0.With medium containing sodium gluconate maximum biomass was produced with 0.1% carbon source by the strain. PHA was however less in this percentage of sodium gluconate such as1.1g/L (3.03%) whereas PHA was greater in 2% sodium gluconate such as10.5% PHA with a maximum of 27.7g/L biomass. Haywood et al., 1990 have reported that sodium gluconate is better carbon source than glucose as growth on glucose may be accompanied by a significant decrease in pH.

During optimization, when the bacterial strain Klebsiella sp. was grown in medium set at pH 5.0, containing o.1% carbon sources such as glucose and sodium gluconate, the strain showed maximum PHA at 24 hour of growth after which the percentage of PHA started declining. The possible reason behind this trend is that in low concentration of carbon source the bacterial cells tend to accumulate the available carbon as reserve but with the passage of time, the cells utilize this reserved carbon resulting in decreased PHA. With 0.1% molasses the same strain showed PHA increasing up to 48 hours possibly because of the nutritional status of the carbon source. At pH 8. The biomass was favored but PHA yield was lower. In the control medium containing 2% glucose biomass increased with the passage of time up to 48 hours whereas PHA was high initially in the cells followed by decline. Medium containing 2% molasses, however, the PHA production trend was consistent with biomass and time reported in recombinant strain of Klebsiella aerogenes utilize sugarcane molasses as the sole carbon source to accumulate PHB at the rate of

approximately 1g of PHB per liter of culture fluid per hour producing a PHB polymer of having molecular weight 2×106. R. eutropha PHB -4 harboring pRKmHS32 (phaC1GPs) accumulated PHA containing small number of MCL-3HA units (3–5 mol%) from sodium gluconate (10wt%) whereas recombinant pseudomonas produced 52% PHA content utilizing 2% sodium gluconate (Taguchi et al., 2001). So Klebsiella sp. produced PHA in glucose optimally at 2% concentration as 23.5g/L biomass, 2.55g/L or 10.89% PHA at 78 hours whereas with sodium gluconate, the optimum concentration found as 0.1% yielding 33.5g/L biomass, 0.5g/L or 7.46% PHA at 56 hours. With molasses as carbon source, biomass production was recorded optimal with 2% supplementation producing 88.7g/L biomass and 8.5g/L or 9.58% PHA, at 78 hours of incubation at 37ºC, pH 7.0. These results were comparable to those of Khardenavis et al., (2009) who obtained PHB up to 31%by utilizing molasses spent wash as carbon source (Chaudharyet al., 2011). FTIR Analysis was performed for PHA produced by Exiguobacterium sp. The bands obtained in FTIR spectra were found comparable to those observed in spectra reported by Hong *et al.,* (1999). Band was observed at wave length 1624.7($cm^{-1}$) corresponds to PHA produced using glucose (control) and at wave 1385. 783 ($cm^{-1}$) with molasses. Results were similar with the spectrum reported by Hong *et al.,* (1999) for polyhydroxybutyric acid. Compound produced by *Exiguobacterium* sp. with different carbon sources thus confirmed as PHB.

## Conclusion

The two strains are capable of producing bioplastics have the potential to overcome the problems associated with conventional plastics derived from finite petrochemical resources. However, the selection of appropriate producer strains as well as cheaper carbon sources along with effective cultivation and extraction processes need to be developed for an effective, useful and purposely commercial scale production of bioplastics.

### References

Akaraonye, E., Keshavarz, T., & Roy, I. (2010). Production of polyhydroxyalkanoates: the future green materials of choice. *Journal of Chemical Technology & Biotechnology*, *85*(6), 732–743.

Chaudhry, W. N., Jamil, N., Ali, I., Ayaz, M. H., & Hasnain, S. (2011). Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. *Annals of microbiology*, *61*(3), 623–629.

Hong, K., Sun, S. Q., Tian, W. D., Chen, G. Q. & Huang, W. (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology*.51: 523-526.

Lee S.P. & Choi J. (1999). Polyhydroxyalkanoates: Biodegradable Polymer. In: Demain, A. L., Davies, J. E., Atlas, R. M. Manual of Industrial Microbiology and Biotechnology, American Society of Microbiology, Washington. pp. 616–627.

Reddy C.S.K., Ghai, R., Rashmi & Kalia V.C., (2003). Polyhydroxyalkanoates: an overview. *Bioresource Technology*, 87, 137– 146.

Rehm B.H. (2010). Bacterial polymers: biosynthesis, modifications and applications. *Nature Review Microbiology*. 8, 578 – 592.

Sohail R., Jamil N., Ali I., & Munir, S. (2020). Animal fat and glycerol bioconversion to polyhydroxyalkanoate by produced water bacteria. *e-Polymers*, 20(1), 92 – 102.

Steinbuchcl A. & Schlegel H.G. (1991). Physiology and molecular genetics of poly (p-hydroxyalkanoic acid) synthesis in *Alcaligeneseutrophus. Molecular Microbiology,* 5, 535 – 542.

Steinbuchel A., Eversloh L.T. (2003). Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal*. 16, 81–96.

Suriyamongkol, P., Weselake, R., Narine, S., Moloney, M., & Shah, S. (2007). Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants—areview. Biotechnology Advances, 25(2), 148–175.

Taguchi, K., Tsuge, T., Matsumoto, K. I., Nakae, S., Taguchi, S., & Doi, Y. (2001). Investigation of metabolic pathways for biopolyester production. *Riken review*, 71–74.

Thellen, C., Coyne, M., Froio, D., Auerbach, M., Wirsen, C., & Ratto, J. A. (2008). A processing, characterization and marine biodegradation study of melt-extruded polyhydroxyalkanoate (PHA) films. *Journal of Polymers and the Environment*, *16*(1), 1-11.

Tripathi, A. D., Yadav, A., Jha, A., & Srivastava, S. K. (2012). Utilizing of sugar refinery waste (cane molasses) for production of bio-plastic under submerged fermentation process. *Journal of Polymers and the Environment*, *20*(2), 446–453.

**Article citation**

Sadaat R. & Jamil N. (2020). Optimization of Bioplastic Production by *Exiguobacterium sp.* and *Klebsiella sp.* using molasses as carbon source. *Journal of Agriculture and Food* 1(1), 22-32.