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# Enumeration of Polyhydroxyalkanoate (PHA) Producing Bacteria from Dairy Sewage Samples

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author KSY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors DBP and MNF managed the analyses of the study. Author RP managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The synthetic polymer plastics have become an integral part of contemporary life. Excess use of plastics and indiscriminate dumping of it in soil and water is polluting the environment. In order to overcome this problem, the production and applications of eco-friendly biodegradable products from microbes are becoming inevitable from the last decade and also are the good alternatives for synthetic polymers.
**Methods and Results:** Polyhydroxyalkanoate producing bacterial strains were confirmed by serial dilution of sewage samples from dairies and pour plating using modified nutrient agar medium with 2% glucose and 0.3% sudan black. Commercial dairy sewage sample from III Dairy showed highest count of PHA producers (3.80 $\log_{10}$cfu/ml) followed by II Dairy (3.68 $\log_{10}$cfu/ml) and I Dairy (3.35 $\log_{10}$cfu/ml). On an average, 70 per cent were PHA producers among TBC of sewage samples.

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**Conclusion:** Dairy sewage sample from III Dairy showed highest count of PHA producers ($3.80 log_{10}$cfu/ml)
**Significance and Impact of the Study:** This study provides importance of polyhydroxyalkanoates and their role against synthetic plastic by enumerating the polyhydroxyalkanoate (PHA) producing bacteria from Dairy sewage samples that can be effectively utilized for the synthesis of bioplastics.

*Keywords: Polyhydroxyalkanoate; synthetic plastic; thermoplastic; biodegradable.*

## 1. INTRODUCTION

"Plastics" are polymers stemming from petro-chemistry. Based on favourable material features such as low density, high resistance, and well-optimized manufacturing processes, plastics are by far the fastest emerging group of materials used for manufacturing of customized items. In this context, plastics are needed for packaging of diverse goods, agriculture, electronics, construction industry, transportation, health care, or the sport and leisure sector. However, based on the limitation of petrochemical resources and the recalcitrance of plastics towards biode-gradation, there is a growing global concern related to traditional plastics of petrochemical origin. Reliable estimates speak about a pile of 8 to 9 x $10^9$ tonnes of plastics that have been made globally in recent decades urgently require proper disposal [1].

Biodegradable polymers offer an alternative, which, due to their technical and economic viability, present a great potential for expansion. Biodegradable polymers are defined as materials that undergo breakage of their chains by the action of microorganisms, resulting in decomposition under specific conditions of pH, humidity, oxygenation, and the presence of catalytically active metals. Biodegradable polymers can be produced from derivatives of fossil sources and renewable natural resources, as well as can be synthesized by bacteria [2]. Among the biodegradable polymers, Polyhydroxyalkanoates (PHAs) are biodegradable polymer family that are produced by diverse microorganisms such as *Pseudomonas putida, Ralstonia eutropha* and *Bacillus megaterium* under nutrient-starved conditions [3]. PHAs are biodegradable linear thermoplastic polyesters, which can be used as alternative polymers to synthetic ones [4]. They are synthesized by different bacterial strains (both Gram positive and Gram negative) cultivated on different carbon sources like sugars, alkanoic acids, alkanes, alkenes and other renewable carbon sources [5]. During the last two decades, PHAs have attained much attention because of their diverse features such as hydrophobicity, elastomeric nature and biodegradability under a wide range of environmental conditions. They also constitute a natural part of renewable carbon cycle and are used as alternatives to synthetic polyesters. Nevertheless, there are still some limitations in their bulk scale production including low yields and high production costs [6].

Some 150 different types of PHAs congeners with different structures (varying side chains or functional groups) and properties had been synthesized using different microbial strains. PHAs can be synthesized though microbial fermentations both at laboratory and pilot plant scales followed by appropriate downstream processing [7]. They are classified according to their chemical unit structure. The polymers containing repeating units with 3–5 carbon atoms are known as short chain length PHA (scl-PHA) and a polymer with repeating units of 6–13 carbon atoms are known as medium chain length PHA (mcl-PHA), while those with more than 13 carbon atoms are known as long chain length PHA (lcl-PHA). A copolymer like poly(3-hydroxybutyrate-co- 4-hydroxyhexanoate) could be produced by simply mixing poly(3-hydroxybutyrate), a scl-PHAs, and poly(4-hydroxyhexanoate), a mcl-PHAs monomers under desirable fermentation conditions. In 1926, French scientist Maurice Lemoigne of Pasteur Institute discovered intracellular accumulation of 3-hydroxybutyric acid polymers in *Bacillus megaterium*, and this was the first report of PHB accumulation in bacteria. These biopolymers are accumulated in the cell to store their utilized carbon sources in depletion of nutrients. PHA has been explored over the years because of its relevant thermal and mechanical properties; furthermore, it can be obtained from renewable resources, degrading enzymatically in different ecosystems such as water, soil, and sludge, among others [8].

Scanty literature is available regarding enumeration of PHA producing bacteria directly as many scientists have done after colonies are formed by staining. The current study was aimed at enumeration of the PHA producing bacteria from various dairy sewage samples directly.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Dairy Sewage Samples

Three Dairy sewage samples were collected in sterile bottles one from student experimental dairy (I Dairy) , Dairy Science college, KVAFSU, Hebbal, Bangalore and other two samples from commercial dairies of Bengaluru (II & III Dairies).

### 2.2 Characterization of Dairy Sewage Samples

The collected dairy sewage samples were subjected to determination of pH, BOD and COD using standard procedure of BIS [9,10]

### 2.3 Enumeration of PHA Producing Bacteria

The dairy sewage samples were serially diluted and pour plated for enumeration of PHA producers. Dairy sewage samples of 11 ml was pipetted and transferred to the sterile 99 ml flask containing physiological saline to make 1st dilution. Further required dilutions were prepared serially using 1st dilution. Serially diluted samples were transferred to labelled sterile petri plates for the enumeration of PHA producing bacteria using sterile pipettes. Sterile molten modified nutrient agar with 2% glucose and 0.3% sudan black [11] maintained at 55˚C water bath was poured to labelled plates containing 1 ml of dilution and mixed thoroughly without spilling the medium. Later the poured agar plates were allowed to solidify. All the poured plates were incubated at 37˚C/48 h by inverting the plates. After the completion of the incubation period, the colonies with dark black coloured colonies were considered as PHA produced and were counted in countable plates ranging between 30-300 and average count was expressed as $\log_{10}$ cfu/ml of the sewage sample.

## 3. RESULTS AND DISCUSSION

All the three dairy sewage samples were neutral with COD on an average of 2944 and BOD of 1400 ppm (Table 1). All the dairy sewage samples from commercial dairies such as I, II and III were subjected to enumeration of PHA bacteria using modified Nutrient agar enriched with 2% glucose and 0.3% sudan black, the plates were incubated at 37˚C/48 hrs (Table 2). I dairy sewage sample showed 3.35 $\log_{10}$cfu/ml of PHA producer followed by II dairy sewage of 3.68 $\log_{10}$cfu/ml and III dairy sewage sample of 3.80 $\log_{10}$cfu/ml. Nehra et al. [12] revealed that the colonies obtained on sterile modified nutrient

**Table 1. Chemical characteristics of collected dairy sewage samples**

| Sample Code | pH | COD | BOD |
|---|---|---|---|
| I Dairy | $6.8^{b}$ | $2100^{b}$ | $800^{b}$ |
| II Dairy | $7.2^{a}$ | $2400^{ab}$ | $1500^{ab}$ |
| III Dairy | $7.2^{a}$ | $3200^{a}$ | $1900^{a}$ |
| **CD (*P* =.05)** | **0.10** | **431.79** | **307.46** |

*Note:*

- *Values are average of three trials*
- *Lower case alphabets as superscript indicate significant difference*
- *CD – Critical Difference*
- *Significantly there was difference in COD and BOD values*

**Table 2. Enumeration of PHA producers of collected dairy sewage samples**

| Sample Code | TBC | PHA |
|---|---|---|
| | ($\log_{10}$cfu/ml) | |
| Dairy I | $5.00^{a}$ (100 %) | $3.35^{a}$ ( 67 % ) |
| Dairy II | $5.07^{a}$ (100 %) | $3.68^{a}$ ( 72.6 % ) |
| Dairy III | $5.32^{a}$ (100 %) | $3.80^{a}$ ( 71.4 % ) |
| **CD (*P* =.05)** | **0.43** | **0.54** |

*Note: Values are average of three trials*

- *Lower case alphabets as superscript indicate significant difference*
- *Total Bacterial Count (TBC) - Pour plated using standard plate count agar and incubated at 37˚C/ 72 h*
- *PHA - Pour plated using Modified Nutrient Agar enriched with 2% glucose and 0.3% Sudan Black and incubated at 37˚C/ 72 h.*
- *Colonies with black colour were counted as PHA producer*

agar medium when flood with sudan black observed black coloured colonies indicating the presence of PHA producers. Many literature identified the PHA producers on nutrient agar after colony formation. But in present research work an attempt was made to use modified nutrient agar with 2% glucose and sudan black (0.3%) and found to be better medium for direct selection of PHA producers.

## 4. CONCLUSION

In the present research work, dairy sewage samples were collected, determined for pH, BOD and COD and further serially diluted and pour plated using 2% nutrient agar with sudan black for the enumeration of PHA producing *Bacillus* spp. Many of the literature revealed that *Bacillus* spp. resulted as potential PHA producing organism by showing positive with Sudan black B staining.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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