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Author (s):	Hamza Abbas Jaffari, Qamar Abbas, Hafiz Zeshan Wadood
Affiliation (s):	Lahore Garrison University, Lahore, Pakistan
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In silico Evaluation of Genes and Proteins Involved in Bacterial BioPlastic Production Pathways

Hamza Abbas Jaffari, Qamar Abbas*, and Hafiz Zeshan Wadood

Department of Biology, Faculty of Basic Sciences, Lahore Garrison University, Lahore, Pakistan

* qamar92@lgu.edu.pk

ABSTRACT

Plastic pollution has become a most pressing environmental issue. The frequent use of plastic products is endangering both fossil fuel resources and the environment. Bioplastics are plastic materials, which have emerged as a suitable replacement and proved more environment friendly than traditional plastics. Among bioplastics, Polyhydroxyalkanoates (PHA) are in demand due to its promising properties and modifications, such as a high rate of biodegradation. Three key genes (phaA, phaB, and phaC) are involved in the biosynthesis of PHA. The current research aims to perform an analysis based on bioinformatic approaches to analyze the genes and their translated proteins in potential PHA producing microorganisms found in the Gen-solution databases. The sequences (DNA and Protein) obtained from PHA-producing microorganisms were analyzed in MEGA X to check their evolutionary history. It was concluded later that the arrangement of genes was highly diverse. The PHA biosynthetic genes belonging to Proteobacteria showed promising phylogenetic relationships. Moreover, the occurrence of their distribution in these organisms suggested that a single gene cluster related to PHA might have been segmented in the form of small groups of genes and transferred among various organisms. The results indicated that the gene and protein sequences were not fully conserved, suggesting that all the genes undergo addition, deletion, or replacement of nucleotides, resulting in gene rearrangement. However, the function of the proteins remained fully conserved, suggesting that the protein function has the highest level of conservation and the gene sequences have the lowest level of conservation. Overall, the results strongly indicate that horizontal gene transfer has a strong influence on the distribution and molecular evolution of the PHA biosynthetic genes.

Keywords: bioplastics, Polyhydroxyalkanoates (PHA), phaA, phaB, phaC, PHA synthesis

INTRODUCTION

Petroleum based plastics have become a necessity and their production has increased dramatically over the past 75 years. Plastic usage is expected to increase in the next 20 years [1]. Synthetic plastics are among the most environmentally harmful substances produced by mankind for instance, occupying the near top position [2].

Since conventional plastics are cheap, versatile, persistent in the environment, and have a poor biodegradation rate, their improper disposal has become an issue globally [3, 4]. A lot of countries have developed special programs toward the discovery of new substances new strategies which has made an attempt to change plastic pollution by making it more environment friendly.

Bioplastics are a special biomaterial made from different microbes, which are cultured under different nutritional and environmental conditions [5]. The number and size of granules, monounsaturated compounds, macromolecular structure, and physicochemical properties vary depending on the organism which is producing them [6–9].

Bacillus megaterium, Alcaligenes latus, Cupriavidus necator, as well as other archaea and cyanobacteria, produce hydroxyalkanoate polyesters as a means of energy or carbon storage materials. After the extensive research on bio-plastics, Polyhydroxyalkanoates (PHAs) were introduced as one of the best alternate options as a greener substitute for conventional plastics [10, 11]. PHAs are produced as a subcellular energy source by many microbes [12]. PHAs have different structural configurations which are a result of the carbon precursor, strain of microbe being used, composition of the media used for growth, fermentation process, and the methods involved in the recovery of PHAs [10, 11, 13]. PHA has up to 150 diverse monomeric structures [14]. Melting temperature, degree of structural order, polymer hydrophobicity, and temperature of glass transition totally depends upon the composition of monomers. [15, 16].

PHAs are ideal storage compounds because they exert negligible increase in osmotic pressure due to their insolubility in the bacterial cytoplasm [17]. The PHA's composition and molecular weight is always determined by the bacterium type and the conditions required for the necessary growth [18].

The synthesis of poly (3-hydroxybutyrate) (PHB) is considered one of the first and best PHA biosynthetic pathway representative. It involves three steps catalyzed by acetyl-CoA acetyltransferase (β-ketothiolase; *pha*A), acetoacetyl-CoA reductase *(pha*B), and PHA synthase *(pha*C) [19, 20].

Various microorganisms have shown this biosynthetic pathway and it has been extensively studied but due to less knowledge of the structural information on the enzymes involved in PHA biosynthesis, the detailed molecular mechanisms involved in the PHA biosynthesis is not that much clear [21, 22].

PHAs are intracellular biopolymers that are of uncommon significance due to their physicochemical, biodegradable, and bio-correspondence properties. These materials can be obtained directly from renewable resources, bioplastics which are petroleum based and can biologically be produced by microorganisms [23]. They can be used for production of food utensils, hygiene items, cosmetic items, glasses, razors, medical surgical clothing, bags, disposable lids, and packaging [24, 25, 26].

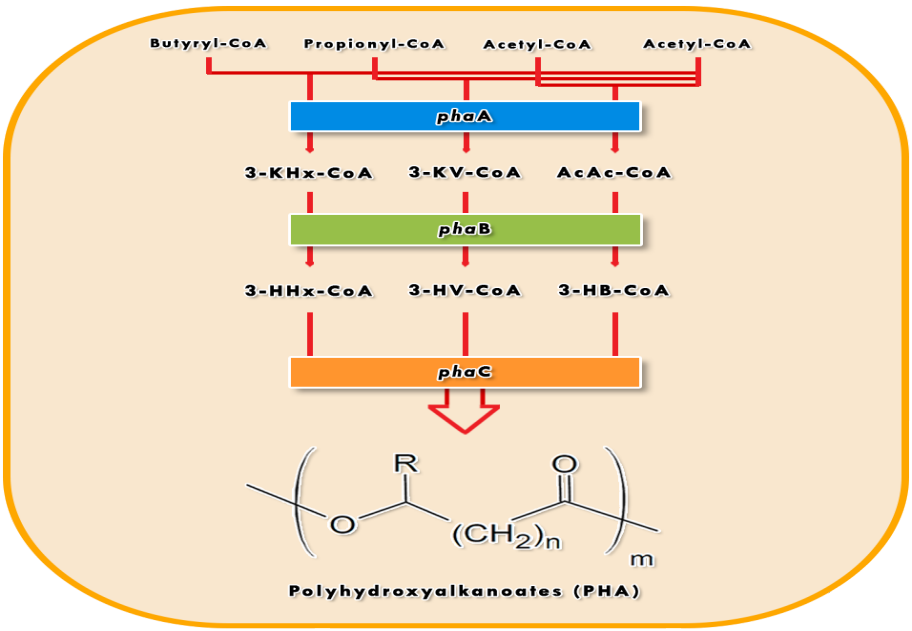

Figure 1. Overview of the Polyhydroxyalkanoate (PHA) metabolism. The arrows represent the metabolic pathways while the boxes show the key enzymes.

There are many challenges regarding production of PHAs which are affecting their commercialization as well. One of them is production cost. The synthetic plastics proved to be much cheaper than PHAs in terms of production costs. Efforts have been made to reduce the production cost by selection of cheap and efficient carbon substrates for the production of PHAs [27].

2. METHODOLOGY

2.1. Data Collection and Filtering

There are three main genes involved in the biosynthesis of PHAs namely *pha*A, *pha*B, and *pha*C. On gen-solution there is an entirely dedicated database for PHA producing organisms obtained by the phylogenetic and statistical analysis of these 3 genes. Gen-solution database provided a lot of relevant and specific information regarding PHA producing microorganisms along with their gene and protein information. The database provided a vast dataset of 23 taxons, 233 organisms, and 381 (*pha*A-111, *pha*B-199, *pha*C-71) genes [28].

All the data obtained from this PHA database was downloaded in software compatible format and relevant information was stored in Microsoft Excel.

In data filtering and refining, all collected data was grouped according to the specific genotype namely *pha*A, *pha*B, and *pha*C, respectively. All sequences belonging to eukaryotic taxa were removed to obtain pure prokaryotic results. The sequence data for all *pha*A, *pha*B, and *pha*C was normalized on the basis of relative size range.

Later, the size of the gene sequences was observed for a common range. For *pha*A the range was about 1100-1200 nt, for *pha*B gene size was ~700-800 nt, and for *pha*C it was approximately 1600-1900 nt. All the sequences that fall in these relative ranges of size were analyzed later.

As a result of the data filtering and refining, microorganisms belonging to *Proteobacteria* were most dominant and were chosen to be analyzed separately.

After analyzing the results of all microorganisms belonging to *Proteobacteria*, alignment files were generated for each genotype (*pha*A,

*pha*B, *pha*C) belonging to each taxon separately namely alpha, beta, and gamma.

2.2. Sequence Alignment using MEGA X

Molecular Evolutionary Genetic Analysis (MEGA) was used for the *in-silico* analysis of the gene and protein sequences as it provides more efficient algorithms for fast and robust analysis [29].

Sequence data was retrieved and refined for sequence alignment. Sequence alignment was performed to find regions of similarity between sequences that may be due to the evolutionary relationships. Individual alignment files were generated for the *pha*A, *pha*B, and *pha*C genes, after every phase of data filtering, containing their respective gene sequences in FASTA format with respect to their genotype.

The alignment files of *pha*A gene, *pha*B gene, and *pha*C gene were aligned independently using the MUSCLE method.

2.4. Phylogenetic Estimation using MEGA X

There are many widely used methods through which the phylogenetic trees are created and analyzed, such as UPGMA, maximum likelihood (ML), neighbor -joining, bayesian inference, and maximum parsimony but in this research, the maximum likelihood method was used to conduct the analysis.

The bootstrap values were set to 1000 replicates. After completion of the process, the constructed phylogenetic tree was displayed.

2.5. Protein Analysis

Protein sequence retrieval was done along with the gene sequences and the same steps were followed to generate alignment files as for the gene sequences. Data filtering of protein sequences was also done along with the gene sequences. The process of sequence alignment and phylogenetic tree generation was same as explained above for the gene sequences.

3. Results

3.1. Variation of Gene Sequences in PHA Producing Microorganisms

Examining the gene and protein sequences after initial dataset filtering and refinement, the results showed that the relationships between the genes, proteins, and their phylogeny varies extremely across the tree of life in prokaryotes. Due to these distinct differences in the gene organization, the

importance in terms of evolution of these genes and clustering them is underlined and is hence, correlated with the concept of evolution [30]**.** Moreover, intra-specie sequence data exhibited diversity among the PHA producing microorganisms.

The results showed that the gene sequences were diversely spread among microorganisms producing PHA. This can be due to gene size being altered by different factors such as nucleotide substitution, gene rearrangements due to presence of transposons, integrases, and recombinases near the gene cluster of PHA biosynthesis. Genes and protein sequences were then filtered according to a preferred relative size range to avoid diverse results and false positives due to variation in gene sizes. The obtained results showed that conserved regions are not there but there are conserved nucleotides which indicated towards conservation of function even if the sequences are not fully conserved.

3.2. Insights in Molecular Evolution of PHA Biosynthetic Genes of *Proteobacteria*

Analyzing the results of initial data filtering and relative size range, it was found that the presence of identical gene clusters is in microorganisms are closely related particularly belonging to *Proteobacteria*. Still there are many instances where the location of these gene clusters contains only a subset of these genes and may be interrupted by genes not involved in PHA biosynthesis. A reason for these genes to be different and not contain conserved regions can be due to different consequences such as insertions or deletions in the genetic sequence that may have caused these gene segments to expand, elongate, and generate a new genetic sequence for the gene in different microorganisms. This suggests that these genes have been linked with horizontal gene transfer and have been shaped extensively by this [31, 32].

3.3. Phylogenetic Analysis of PHA Biosynthetic Genes in *Alpha, Beta,* and *Gamma Proteobacteria*

Conserved gene pattern was found in *Proteobacteria*, particularly *alpha, beta,* and *gamma Proteobacteria*. The gene clusters appeared to be both common and conserved in these. This investigation primarily, focused on determining the phylogenetic relationship of these PHA producers but there was no intention to study how these PHA gene clusters were produced or how they got distributed among different organisms.

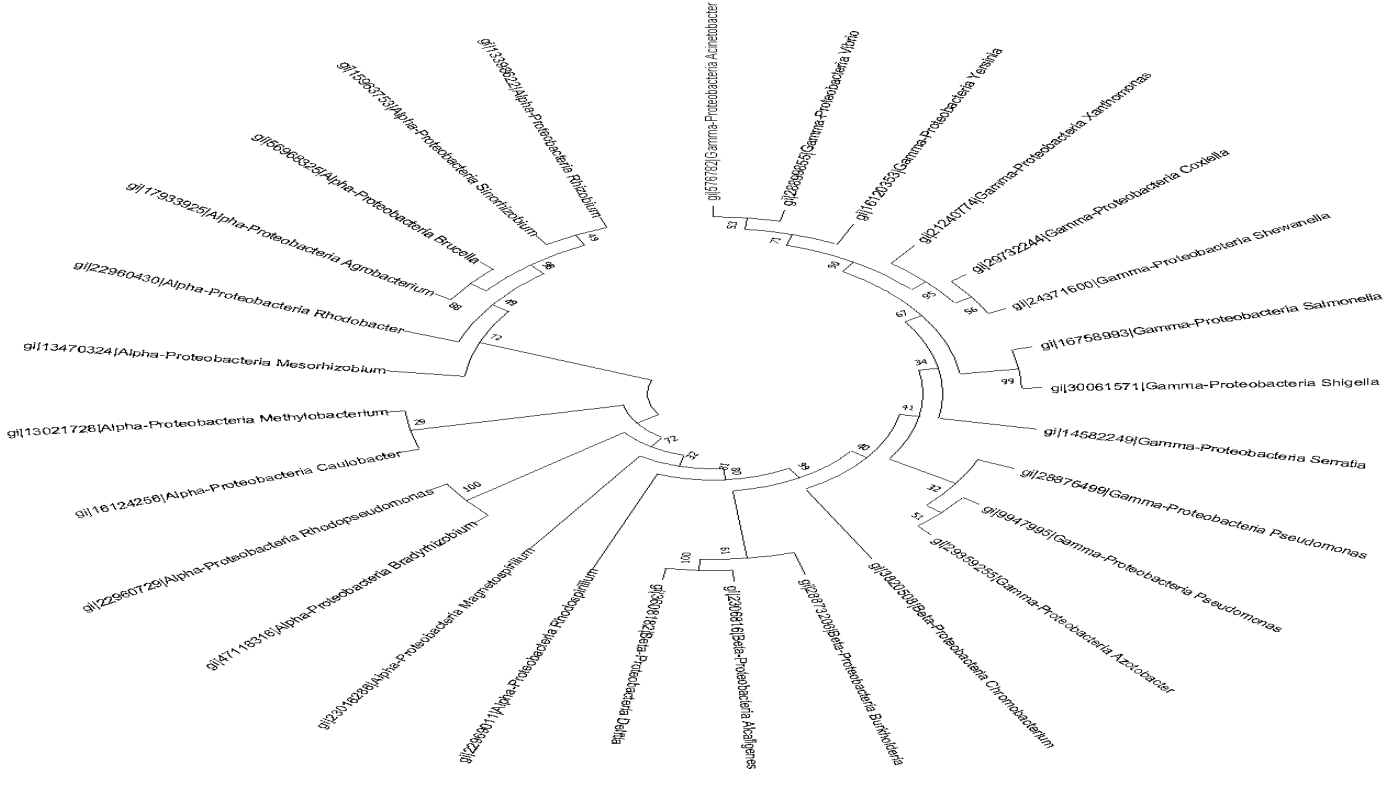

Figure 2. The Tamura-Nei model and the Maximum Likelihood approach were used here to determine the phylogeny of *pha*A, *pha*B and *pha*C genes of microorganisms belonging to *Proteobacteria*, represented by a consensus tree generated from 1000 replicates of bootstrap values. MEGA X was used to undertake evolutionary analysis. **(a)** *pha*A: This study included 28 nucleotide sequences with the final dataset having a total of 2172 positions.

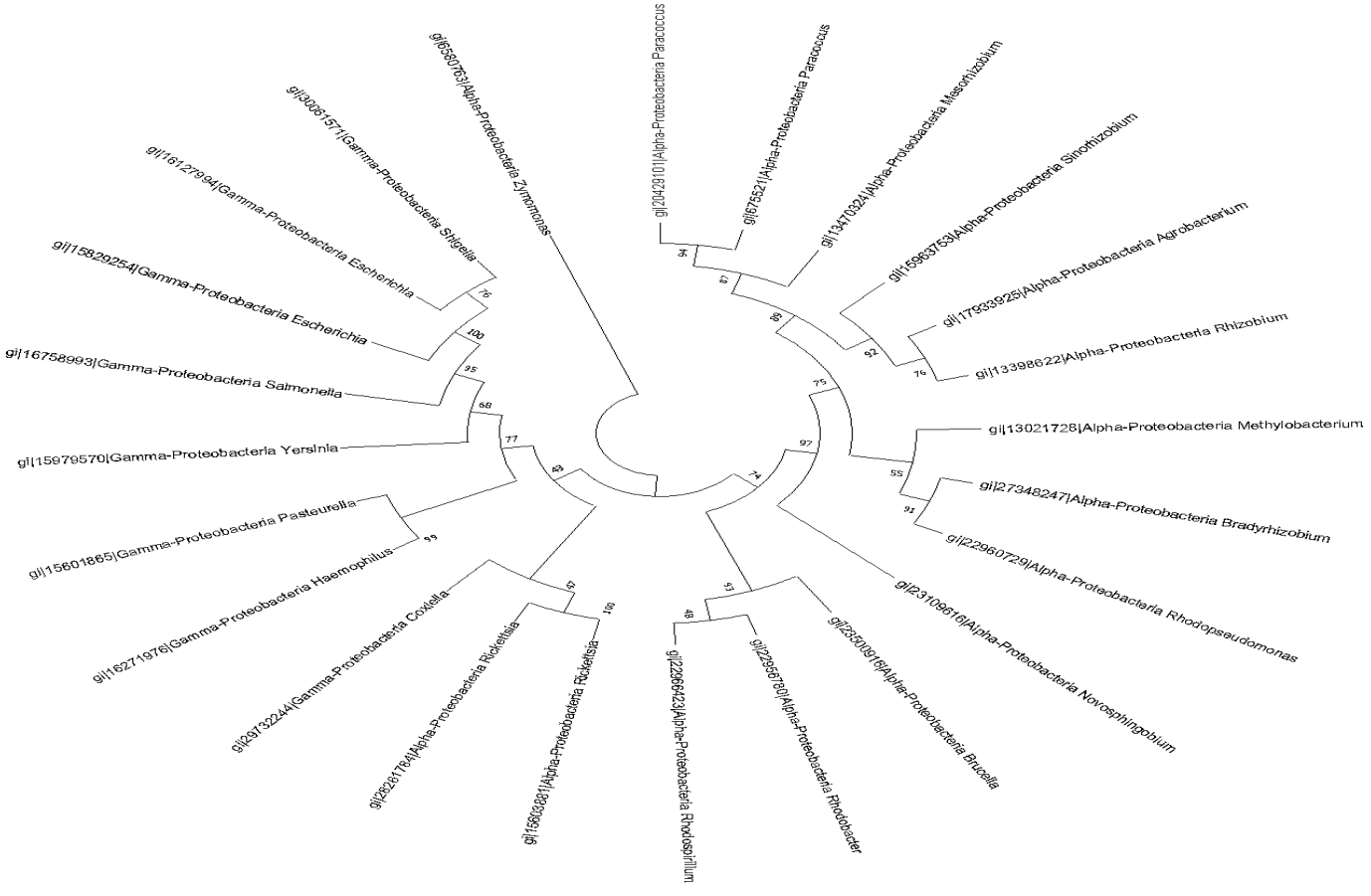

Figure 2b. *pha*B: This study included 24 nucleotide sequences with the final dataset having a total of 872 positions

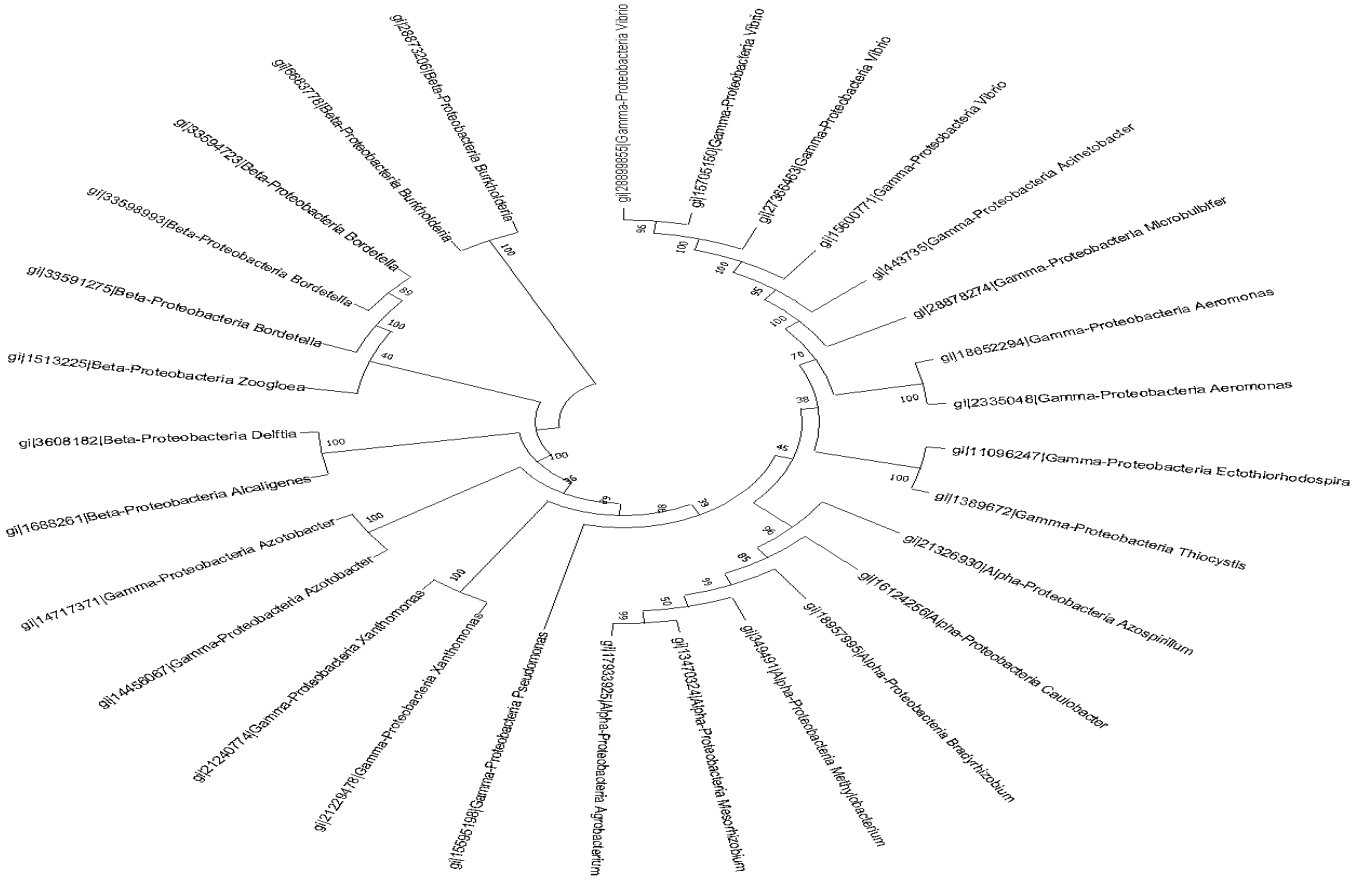

Figure 2c. *pha*C: This study included 29 nucleotide sequences with the final dataset having a total of 2456 positions

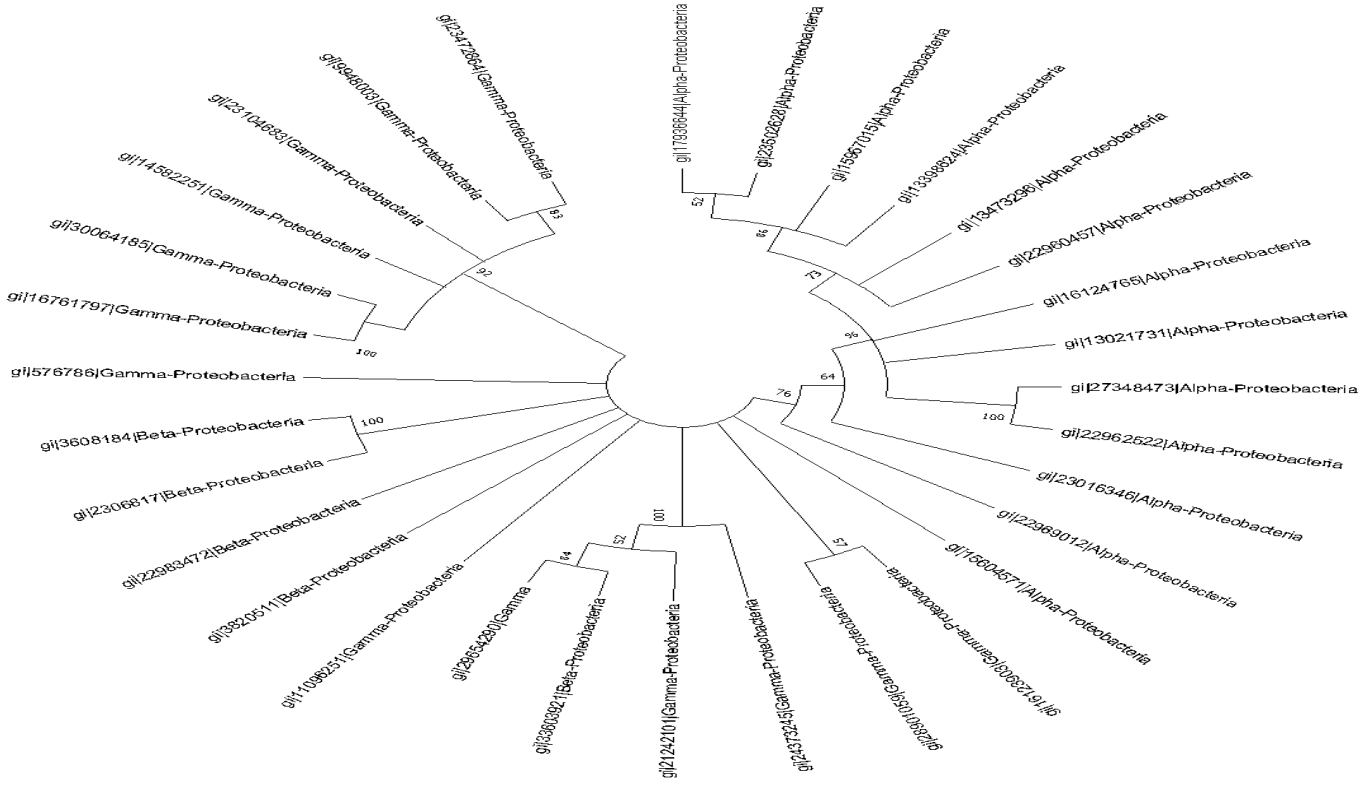

Figure. 3. The JTT matrix-based and the Maximum Likelihood approach were used here to determine the phylogeny of *pha*A, *pha*B and *pha*C proteins of microorganisms belonging to *Proteobacteria*, represented by a consensus tree generated from 1000 replicates of bootstrap values. MEGA X was used to undertake evolutionary analysis **(a)** *pha*A: This study included 31 nucleotide sequences with the final dataset having a total of 712 positions

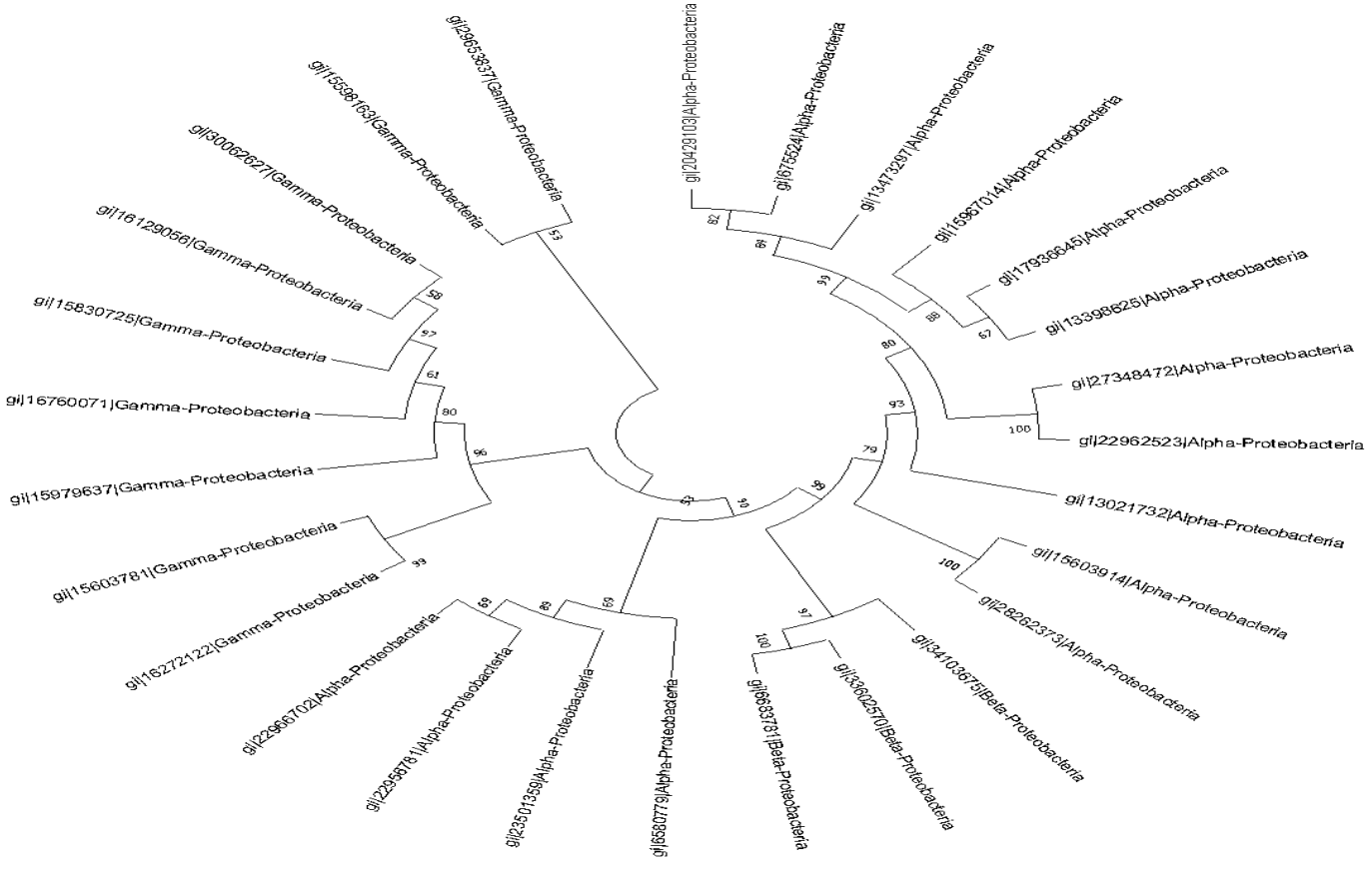

Figure 3b. *pha*B: This study included 27 nucleotide sequences with the final dataset having a total of 270 positions

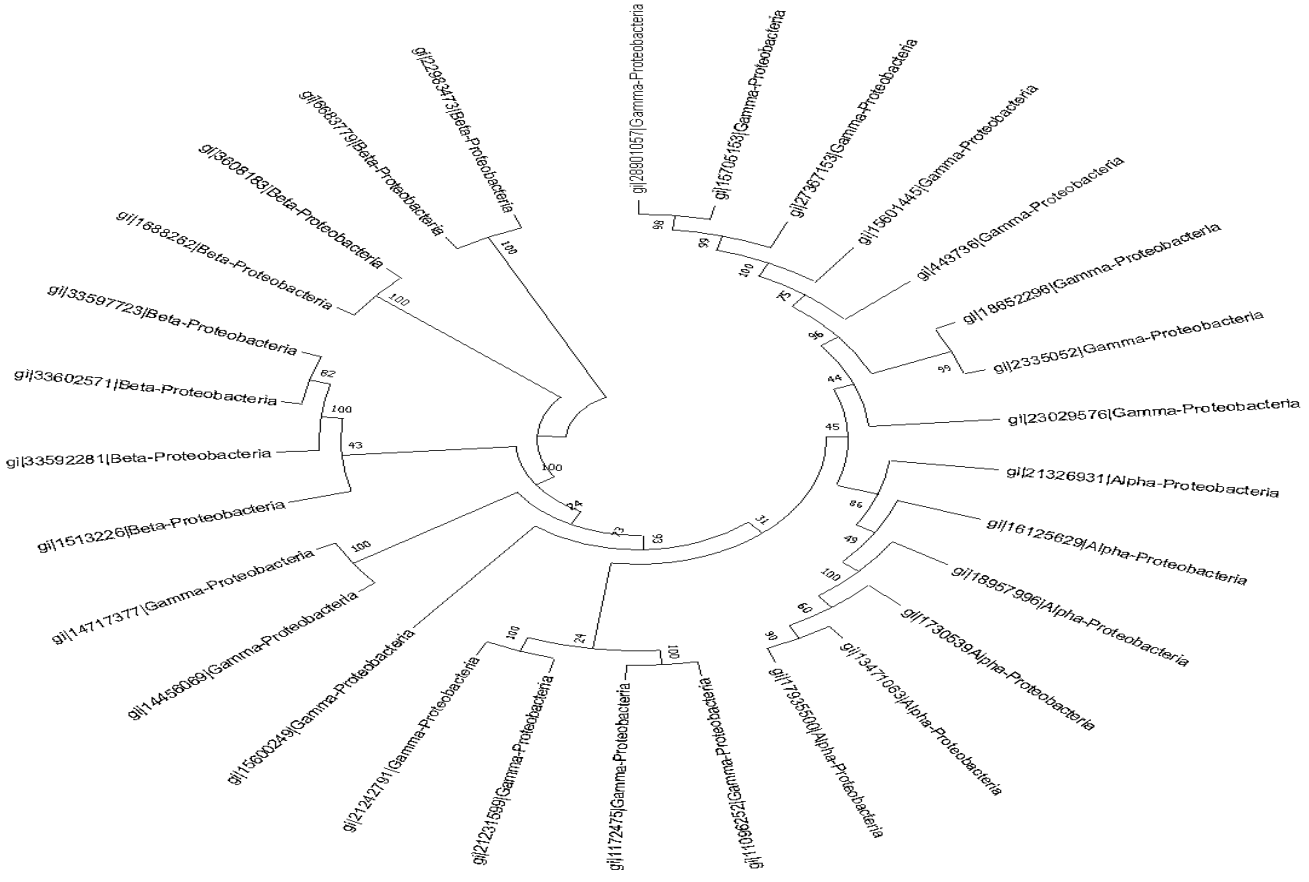

Figure 3c. *pha*C: This study included 29 nucleotide sequences with the final dataset having a total of 794 positions

It has been demonstrated in previously conducted studies that genes that are involved in the metabolism of PHA has experienced independent evolution and have a greater degree of difference as compared to the other genes [32]. The results displayed that the majority of the PHA biosynthetic genes were found in the microorganisms belonging to the group of *alpha, beta,* and *gamma Proteobacteria.*

3.4. Comparative Phylogenetic Analysis of Proteins involved in PHA Biosynthesis

After examining the results of protein sequence analysis and comparing them with the results of gene sequence analysis, it was evident that the conservation of protein sequences was higher than that of the genes. The alignment sessions performed after all phases of data filtration showed that there was higher degree of conservation among protein sequences in comparison to gene sequences.

4. DISCUSSION

The PHA pathway is important for free-living strain's environmental fitness, as evidenced by the extensive maintenance of genes clustered in microorganisms living in a variety of different habitats [33, 34]. No significant differences in genes content were found in *Proteobacteria,* this suggests that environment is a better and greater factor in determining the PHA gene clusters than the phylogenetic origin. Furthermore, it is believed that genes and their clustering make functionally related metabolic genes to shift by horizontal gene transfer (HGT) easier for organisms.

Phylogenetic studies indicated that once acquired, PHA genes have never been lost during the course of evolution. Clearly, the stepwise and progressive novel PHA gene addition and disruption has caused the cluster size to increase. The clusters are made up of orthologous genes found in closely related or unrelated species.

PHA genes and their neighbour's order proved to be diverse and conserved. In closely related bacterial species of *Proteobacteria*, adjacent genes were kept near to PHA-associated genes clusters. This diversification in the rearrangements strongly suggested that genome relocation events had a substantial role in maintenance of the biosynthetic genes namely *pha*A, *pha*B, and *pha*C [30, 35].

The *pha*A, *pha*B, and *pha*C genes may have been acquired through horizontal gene transfer from a recent common ancestor throughout evolution, based on gene order conservation and phylogeny. Considering all these observations, it can be concluded that the PHA biosynthetic genes were probably originated through of horizontal gene transfer or native gene relocation [30].Majority of the products of surrounding genes in the vicinity of *pha*A, *pha*B, and *pha*C are relatively stable and essential for cell survival [36, 37]**.** The results of genes alignment and phylogenetic analysis showed that these genes have been continuously maintained throughout all genomes, meaning that they have been successfully transferred and conserved in the descendants via horizontal gene transfer events for optimal cell machinery function.

After analyzing the results of both genes and proteins analysis consisting of sequence alignment and phylogenetic analysis, it was hypothesized in the current study that during the course of natural selection in evolution in an ancestor, PHA-related genes (particularly *pha*C) were transferred along with surrounding genes and established long-term relationships for maintenance. On the other hand, this can also be a result of horizontal genes transfer in order to improve the genetic stability and maintenance in the microorganisms [38].

4.1. Conclusion

This study focused on the production of polyhydroxyalkanoates (PHA). It showed that there are three key biosynthetic genes which are involved in the production of PHA, namely phaA, phaB, and phaC. This research analyzed the methods of detecting these genes in different microorganisms, by enabling them to become a potential PHA producer. The gene and protein analysis along with the evolutionary history of PHA biosynthetic genes showed that horizontal gene transfer and rearrangement of the nucleotide sequences is a major contributors to the evolution of PHAs. Gene and protein sequences are not fully conserved across al PHA-producing microorganisms; however, there are conserved nucleotides which indicate that there may have been instances of gene rearrangement due to the addition, deletion or replacement of a nucleotide. The genes still coded for the relative proteins involved in the biosynthesis of PHAs show that even though the sequences may not be adequately conserved, yet the functions remain completely conserved. The highest level of conservation found was of the protein function, then the protein structure, followed by the protein

and gene sequences. Microorganisms belonging to *Proteobacteria* appeared to be the closest in terms of the phylogeny studied regarding PHA producers. This indicates that these microorganisms are more conserved than those belonging to other families.

4.2. Implications and Recommendations

The study of these genes and proteins sequences is essential as it reflects that the sequences have undergone mutations or variations at certain points, while being transferred from one microorganism to the other. Even so, they still code for the same protein and regardless of the variations in the gene and protein sequences, the protein structure and the protein function remain conserved. The study indicates that many microorganisms which possess the key genes required to produce PHA. Some are commonly found in the environment and can easily be cultured in the lab using cheap carbon and nitrogen sources as growth factors. This depicts that the microorganisms in possession of these three key genes are potential PHA producers which can be studied further for a cost-effective way of PHA production.

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