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Whole Genomic Sequencing and Analysis of *Bacillus subtilis* PWA: Linking Plastic Bioconversion to PHA Production

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ABSTRACT

Plastic pollution requires innovative microbial solutions for plastic valorisation into sustainable bioproducts, such as intracellular PHA, during stressed conditions. This study reports the whole genome sequencing (WGS) and annotation of *Bacillus subtilis* PWA, using plastics for biopolymer PHA production. High throughput genome sequencing using Illumina NovaSeq 6000 gave a total of 983,667 raw reads with 114x average genome coverage. Genome assembly (4.3 Mb draft genome with 43.18% GC content) was submitted to NCBI database under the assembly number GCA_047314825.1. Approximately 4,517 genes (4,191 protein-coding) were annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Functional annotation revealed genes associated with plastic degradation (*alkB1*, *alkB2*, *alkB3*, and *CYP153*) and PHA biosynthesis (*phaA*, *phaB*, *phaC*, *phaJ*, *phaZ*). The identification of genes concerned with survival and adaptation, for instance, osmoprotectant uptake (*opuAA*, *opuAB*, *opuCA*, *opuCC*), betaine biosynthesis (*betB*, *opuD*), and choline metabolism (*natAB*) supports the ability of strain PWA to survive in extremely stressed environments. Genome was visualized using Proksee CGViewer, while protein structure was visualized using AlphaFold. Metabolic pathways studied in the Kyoto Encyclopaedia of Genes and Genomes (KeGG) demonstrate modules for hydrocarbon degradation and PHA biosynthesis. The findings of this study including the genomic repertoire underscore the circular bioeconomic potential of the strain PWA for large-scale bioplastic production through the bioconversion of plastic wastes. Future studies should focus on metabolic engineering of PHA operon for optimized and volumetric production.

Keywords: gene annotation, genome assembly, illumina, metabolic pathway, protein visualization, whole genome sequencing (WGS)

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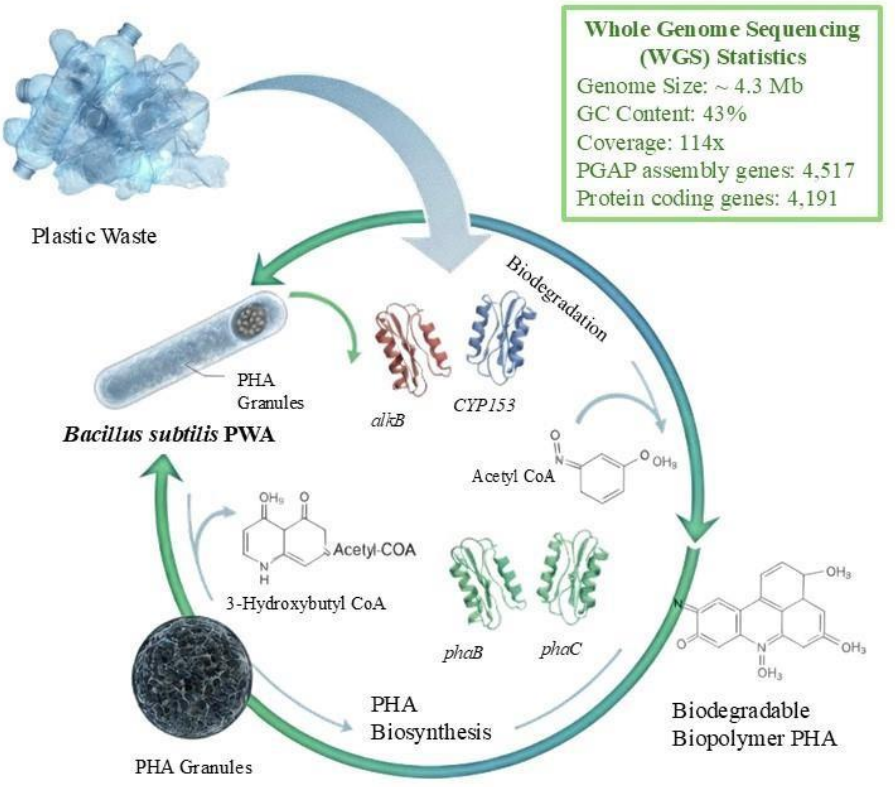
GRAPHICAL ABSTRACT



1. INTRODUCTION

Research in genomics has led to an in-depth understanding about the various metabolic pathways that govern an organism's life. The entirety of gene complements in an organism's genome can familiarize a scientist with the particular organism's metabolic capacity. Studies dealing with gene sequencing, annotation, and analysis provide the necessary knowledge required for the manipulation of biosynthetic pathways for enhanced yield, co-polymer production, and other related aspects [1]. In bacteria, genome is housed in a supercoiled circular chromosome, while the G+C content remains relatively consistent within a genus or specie [2]. Typically, bacterial genomes undergo duplications, transpositions, insertions,

inversions, deletions, mutations, and recombinations. However, only genes with desirable traits that impart a competitive advantage during natural selection are conserved over time [3].

Next-generation sequencing techniques, such as polony sequencing, sequencing by synthesis, ligation sequencing, and single-molecule DNA sequencing have come to dominate the global market. Illumina sequencing employs the synthesis method - DNA amplification on slides for the creation of clusters through primer attachment and depolymerases, aided by fluorescently tagged reversible-terminator nucleotides [4, 5]. A genome sequencing project usually has an error rate of 1 per 103~105 bases. Sequenced genomes are annotated by the comparison of sequences to similar genes with well-known roles. Annotated bacterial genome datasets are available in multiple repositories [6]. These genome repositories can aid in the identification and annotation of unidentified genes using various software, such as BLAST. The identification and annotation of novel genes, however, poses an issue that can potentially be resolved by anticipating gene function and inferring the functional and structural expression profile [7-10]. Microarray analysis can be employed to identify genes that express themselves under different experimental conditions. Microarray technologies have significant sensitivity, specificity, and discriminatory power.

The introduction of fabrication and robotics have led to developments in the field of bioinformatics [11, 12]. High-density bead arrays, suspension bead arrays, and three-dimensional arrays have further led to increased efficiency. A number of platform independent, bioinformatics software tool packages are available that aid in genome mining for the identification of gene clusters, such as *anti*biotics & *S*econdary *M*etabolite *A*nalysis *Sh*ell (antiSMASH) [13].

This study describes the whole genome analysis of previously isolated bacterial strain PWA – capable of biopolymer production using plastic – in order to identify the major genetic elements conferring unique traits. The metabolic pathways outlining plastic bioconversion to biopolymers, such as PHA, are also identified.

2. METHODS

2.1. Whole Genome Sequencing (WGS) of Strain PWA

Previously isolated bacterial strain *Bacillus subtilis* PWA – capable of

producing biopolymer PHA using plastic as the sole carbon source [14] was selected for whole genome sequencing (WGS). Illumina NovaSeq 6000 sequencer (Illumina, San Diego, USA) [4] was used for WGS by GetGenome – a non-profit organization – that provides access to genomics technology [15].

2.1.1. Sample Submission. Pure cultures of bacterial strains were grown on N. Agar media and sent to GetGenome [15]. *Bacillus subtilis* PWA was grown on N. Agar broth and harvested. Cells were re-suspended in tubes with DNA/RNA Shield (Zymo Research, USA) and processed according to the submission procedures outlined by MicrobeNG. The prepared samples were sent to MicrobeNG for sequencing.

2.1.2. DNA Processing. Cell suspension 40-50 µL was lysed using 120 µL TE buffer containing 0.1 mg/mL lysozyme and 0.1 mg/mL RNase A (ITW Reagents Barcelona, Spain). The suspension was incubated for 25 min at 37°C. After 25 min, 0.1 mg/mL Proteinase K (VWR Chemicals, Ohio, USA) and 0.5% SDS (Sigma-Aldrich, Missouri, USA) were added and the suspension was incubated at 65°C for another 5 min. An equal volume of SPRI beads was used for the purification of genomic DNA and the solution was re-suspended in EB buffer (10 mM Tris-HCL, pH 8.0) [16, 17]. The extracted DNA was quantified using Quant-iT dsDNA HS kit (ThermoFisher Scientific) assay in an Eppendorf AF2200 plate reader (Eppendorf UK Ltd) and diluted as appropriate [18].

2.1.3. Illumina Sequencing. Nextera XT Library Prep Kit (Illumina, San Diego, USA) was used for the preparation of genomic DNA libraries with a couple of modifications: input DNA increased two-fold and PCR elongation time increased to 45 sec [19]. Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland) was used for DNA quantification and library preparation [20]. A 250 bp paired end protocol was adapted for library sequencing using Illumina NovaSeq 6000 (Illumina, San Diego, USA) [4].

2.2. Genome Analysis and Annotation

Raw reads and assembled sequences were obtained through WGS. Trimmomatic 0.30 was used for adapter trimming of reads, with a sliding window quality cut-off of Q15 [21]. SPAdes 3.15.4 was used for de novo assembly of samples [7], while Prokka 1.11 was utilized for contig annotation [22]. The 16S rRNA gene was identified by inspecting homology

using BlastN. The sequences were submitted to NCBI GenBank. The evolutionary relationship of strain PWA with related taxa was mapped using MEGA 10.1.7 [23]. Raw reads and assembled sequences were submitted to NCBI database Sequence Read Archive (SRA). Genome was annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [24]. Metabolic pathways for PHA production and plastic degradation were extracted using Kyoto Encyclopaedia of Genes and Genomes (KeGG) [25]. Following GenBank annotation, Porksee CGViewer was used for the construction of circular genome, utilising reference bacterial genomes [26]. AlphaFold database was utilized for the visualization of protein structure [27].

3. RESULTS AND DISCUSSION

3.1. Whole Genome Sequencing of Bacterial Strain *Bacillus subtilis* PWA

Illumina NovaSeq 6000 was used for the WGS of bacterial strain *Bacillus subtilis* PWA. The summary of reads is provided in Table 1 which shows the strain ID, genome coverage, and number of reads. Table 1 also describes the summary of raw assembly statistics. The total number of bases, GC content (%), N50, N75, L50, and L75 for all genome assemblies are provided.

Table 1. Raw Reads Summary and Assembly Statistics for the Whole Genome Sequence of Bacterial Strain *Bacillus subtilis* PWA

Raw Reads Summary			
Median insert size	698		
Mean coverage	114.483		
Mean coverage excluding 0s	114.494		
Number of reads	983667		
Number of reads w/ insert size > 300	855934		
Raw Assembly Statistics			
# contigs (>= 0 bp)	102	GC (%)	43.18
# contigs (>= 1000 bp)	64	N50	110781
Total length (>= 0 bp)	4141436	N75	66877
Total length (>= 1000 bp)	4128032	L50	12
Largest contig	291256	L75	24

3.2. 16S rRNA Gene Sequencing

Strain PWA – gram positive bacilli – isolated from the produced water

was identified as *Bacillus subtilis*. The 16S rRNA gene sequence (1456 bp) of *Bacillus subtilis* PWA was identified and submitted to NCBI GenBank under accession number PQ608827. The evolutionary relationship of bacterial strain with closely related taxa was computed (Figure 1).

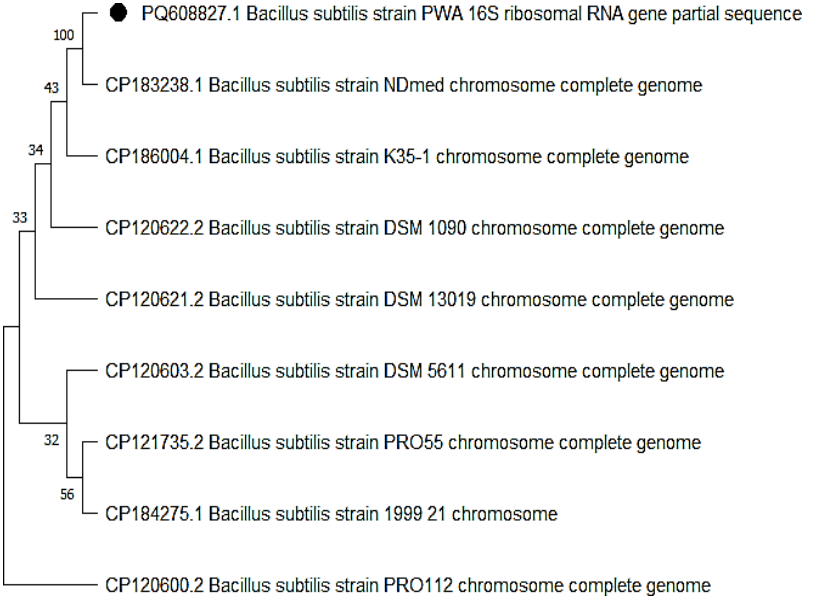


Figure 1. Evolutionary Relationship of Closely Related Taxa with *Bacillus subtilis PWA*.

Neighbor-Joining approach was used to infer the evolutionary history in terms of the number of nucleotide substitutions per site. A total of 500 replicates were taken for Bootstrap consensus tree and branches corresponding to partitions reproduced in less than 50% replicates were collapsed. The optimal tree is presented to scale, and the branch lengths correspond to evolutionary distances determined with the Maximum Composite Likelihood approach. The analysis contained 9 nucleotide sequences while the codon sites given were first, second, third, and noncoding. All ambiguous sites in each sequence pair were removed (using the pairwise deletion option). The entire dataset had a total of 118 sites.

3.3. Submission of *Bacillus subtilis* PWA Whole Genome to NCBI Database

The raw reads (BioSample SAMN44802318) and assembled sequences

(JBLBWK000000000) were submitted to NCBI under BioProject PRJNA1187766 as assembly GCA_047314825.1. Table 2 outlines the assembly statistics and annotation details for the WGS of strain PWA.

Table 2. Assembly Statistics and Annotation Details for the WGS of *Bacillus subtilis* PWA

Genome size	4.3 Mb
Number of contigs	93
GC percent	43
Genome coverage	114x
PGAP assembly genes	4,517
Protein coding genes	4,191

3.4. Genome Analysis and Annotation

The WGS analysis of *Bacillus subtilis* PWA provided significant insights into the various metabolic processes involved in plastic biodegradation and bioconversion to PHA. The genome analysis exhibited the genes related to plastic degradation, PHA production, EPS production, biosurfactant production, and plant growth promotion. Genes involved in survival mechanisms, such as salt tolerance, were also identified (Table 3).

Table 3. Notable Secondary Metabolic Genes Present in WGS of *Bacillus subtilis* PWA

Processes		Gene Name	Gene Expression (Protein/Product)	Locus ID
Stress Tolerance	Osmo-protectant	*opuAA*	glycine/proline betaine ABC transporter ATP-binding protein OpuAA	ACL9SR_RS07110
		opuAB	glycine/proline betaine ABC transporter permease subunit OpuAB	ACL9SR_RS07105
		opuBA	Osmo-protectant uptake	ACL9SR_RS18825
		opuBB	Choline ABC transporter permease OpuBB	ACL9SR_RS18820
		opuBC	Choline ABC transporter permease OpuBC	ACL9SR_RS18815
		opuBD	Choline ABC	ACL9SR_RS18810

Processes		Gene Name	Gene Expression (Protein/Product)	Locus ID
			transporter permease OpuBD	
		opuCA	Osmo-protectant ABC transporter OpuCA	ACL9SR_RS18875
		opuCB	Osmo-protectant ABC transporter permease	ACL9SR_RS18870
		opuCC	Osmo-protectant uptake transporter system OpuCC	ACL9SR_RS18865
	Glycine Betaine Biosynthesis	*betB*	Betaine-aldehyde dehydrogenase	ACL9SR_RS22045
		OpuD	Glycine-betaine transporter	ACL9SR_RS17080
	Proline Biosynthesis	*proB*	Glutamate 5-kinase activity	ACL9SR_RS05335
		proC	Pyrroline-5-carboxylate reductase	ACL9SR_RS00370
		OpuE	L-proline transporter	ACL9SR_RS1232
	Choline Biosynthesis	*opuBC*	Choline ABC transporter OpuBC	ACL9SR_RS18815
		natA	ATPase component NatA	ACL9SR_RS07230
		natB	Permease component NatB	ACL9SR_RS07225
		gbsB	Choline dehydrogenase	ACL9SR_RS22050
PHA Production		*accA*	Acetyl-CoA carboxylase	ACL9SR_RS08380
		phaA; accB	Acetyl-CoA C-Acyltransferase	ACL9SR_RS00660
		phaB;mmgB	3-hyroxybutyryl-CoA dehydrogenase	ACL9SR_RS00560
		phaC	PHA Synthase	ACL9SR_RS20835
		phaJ	Enoyl-CoA hydratase	ACL9SR_RS02320
		phaZ	PHA Depolymerase	ACL9SR_RS02290
		fabD	Malonyl-ACP	ACL9SR_RS13755
		fabF	Beta-ketoacyl-ACP synthase II	ACL9SR_RS14995
		fabG	3-ketoacyl-ACP reductase	ACL9SR_RS13760
		fabH	Beta-ketoacyl-ACP synthase III	ACL9SR_RS14990
		fabI	Enoyl-ACP reductase FabI	ACL9SR_RS15200
		fabZ	3-hydroxyacyl- ACP dehydratase	ACL9SR_RS13875
		fadE	Acyl-CoA	ACL9SR_RS20845

Processes	Gene Name	Gene Expression (Protein/Product)	Locus ID
		dehydrogenase	
	fadD; mbcS	Acyl-CoA synthetase	ACL9SR_RS08185
Plastic Biodegradation	*alkB1*	4-hydroxy-phenylacetate 3-monooxygenase	ACL9SR_RS02655
	alkB2	Alkane monooxygenase	ACL9SR_RS02655
	alkB3	Alkane monooxygenase	ACL9SR_RS10285
	CYP153	Cytochrome P450; alkane hydroxygenase	ACL9SR_RS17025

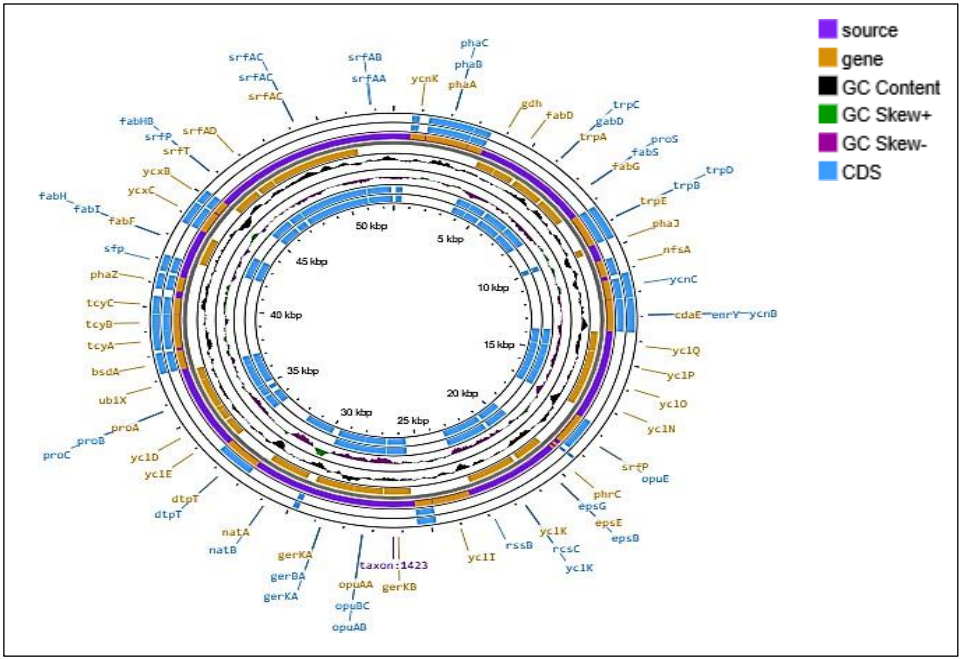


Figure 2. The circular genome of *Bacillus subtilis* PWA obtained through Proksee CG viewer

Plastic degradation genes *alkB1*, *alkB2*, *alkB3*, and *CYP153*, as well as various PHA biosynthesis genes *phaC*, *phaR*, and *phaZ*, were also identified. Genome analysis also revealed the presence of several tolerance genes – genes for osmo-protection, glycine-betaine biosynthesis, proline biosynthesis, and choline biosynthesis. Genes for ABC transporter system (*opuBA*, *opuCA*, *opuCC*) employed for the transport of osmoregulators and energy coupling were found in *Bacillus* genome. Proksee CG viewer was

used to visualize the circular genome of *Bacillus subtilis* PWA (Figure 2).

Track 1,2 were obtained through NCBI annotated contigs. Track 3, 4 represent GC content and positive, negative GC skew. While track 5-8 represent Prokka annotated genes.

Genome analysis of *Bacillus subtilis* PWA showed the presence of various genes associated with PHA biosynthesis and plastic degradation. Various enzymes including laccases, lipases, hydroxylases, esterases, and reductases have been reported to play a critical role during plastic biodegradation [28].

The biodegradation of petrochemical derived polyethylene is mediated by genes *alkB* and *CYP153*, which catalyse the biodegradation of medium and short chain length alkanes [29, 30]. Polyethylene degradation, in particular, has been attributed to *alkB* genes *(alkB1, alkB2, alkB3, alkB4,* and *alkB5)* [11]. The protein product of gene *alkB* is visualized using AlphaFold (Figure 3).

The *alkB* gene encodes alkane monooxygenase, while cytochrome P450 alkane hydroxylase is encoded by *CYP153* gene [31]. Lakshmi et al. also reported the presence of *alkB* gene in LDPE degrading *Pseudomonas* sp. [30]. Polycyclic aromatic hydrocarbon degradation was studied using KeGG database. Figure 4 depicts the degradation pathways for polyethylene, polyethylene terephthalate, and styrene, among others.

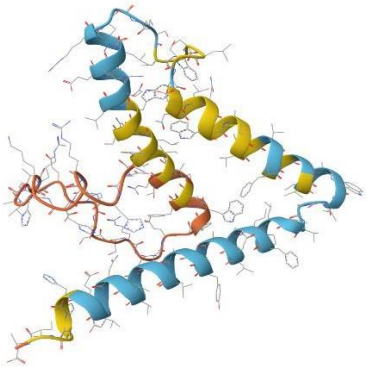


Figure 3. Alkane Monooxygenase Protein of *Bacillus sp.* Encoded from Gene *alkB* (Protein Structure Extracted from AlphaFold Database; https://alphafold.ebi.ac.uk/entry/A0A238F166).

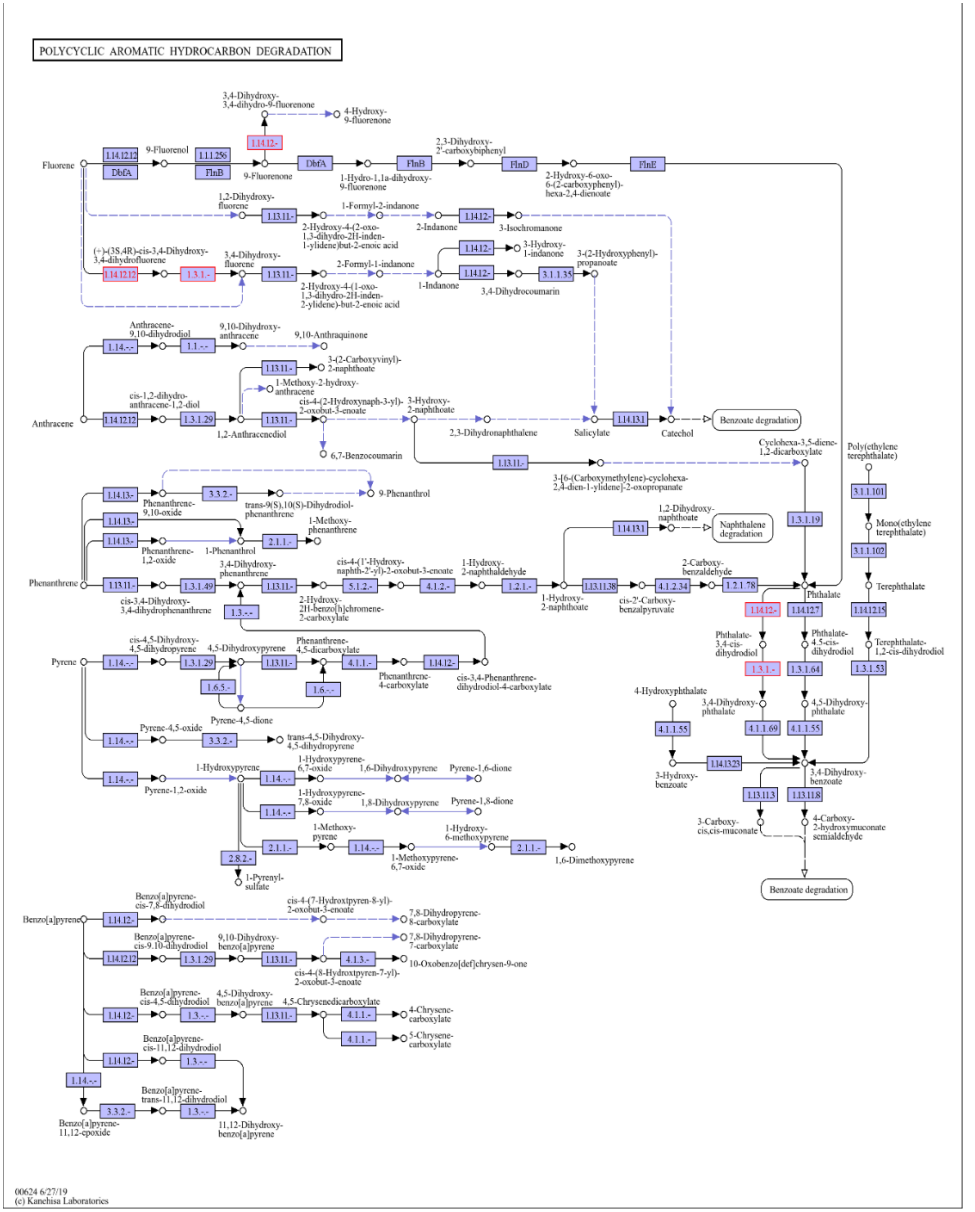


Figure 4. Polycyclic Aromatic Hydrocarbon Degradation Depicting Degradation Pathways for Polyethylene, Polyethylene Terephthalate, Styrene, etc. (Extracted from KeGG).

Genome analysis also showed the presence of PHA biosynthetic genes *phaC*, *phaZ*, and *phaR*. In a similar study, Ebu et al. explored the genome

assembly of PHA producing *Bacillus cereus* strain and reported the presence of several PHA biosynthetic genes, including *phaP*, *phaB*, *phaR*, *phaZ*, *phaQ*, *phaC*, and *phaA* [32]. PHA depolymerase is encoded by gene phaZ, involved in the degradation of intracellularly stored PHA. Literature review shows that *phaZ* is the primary enzyme in PHA degradation, however, it is not typically the primary enzyme for synthetic plastic degradation. Whereas, PHA synthase protein is encoded by gene *phaC* and is primarily involved in PHA synthesis. PHA operon contains various genes, among which PHA synthase *phaC* gene is absolute for PHA production. On the basis of substrate specificity and subunits, PhaC (expressed form of *phaC* gene) is categorized into four classes. Class I synthases, such as those in *Ralstonia eutropha*, encode PhaC synthase (61-73 kDa) which prefer CoA thioesters of R-3-hydroxyacids (3-5 carbon atoms) and form scl-PHA. Whereas, class II synthases, encoding PhaC synthase that prefers R-3-hydroxyacids CoA thioesters with 6-14 carbon atoms, are found in *Pseudomonas aeruginosa* and form mcl-PHA. Class III synthases encode two subunits, namely PhaC (40 kDa) and PhaE (40 kDa), which produce scl-PHA and are found in *Allochromatium vinosum*. Class IV synthases also encode two subunits, namely PhaC (40 kDa) and PhaR (20 kDa). Class IV synthases are only found in *Bacillus* species. Both specific and generic primers are used for the amplification of PhaC from classes III and IV and classes I and II, respectively. A similar study by Sachan et al. reported the presence of *phaR*, *phaB*, and *phaC* genes in *Bacillus paranthracis* strain RSKS-3 [33]. Protein products of genes *phaC and phaZ* are visualized using AlphaFold (Figure 5, 6).

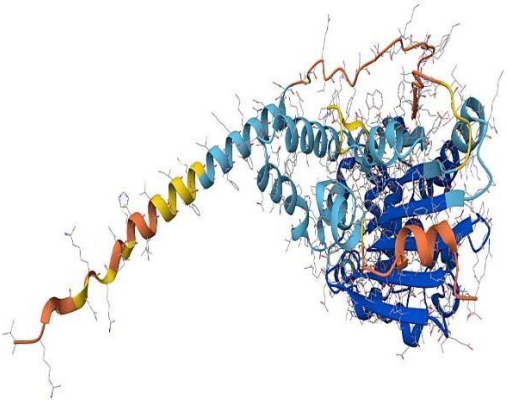


Figure 5. PHB Synthase Subunit *PhaC* Protein of *Bacillus subtilis* Encoded

from Gene *phaC* (Protein Structure Extracted from AlphaFold Database; https://alphafold.ebi.ac.uk/entry/I4EBJ0).

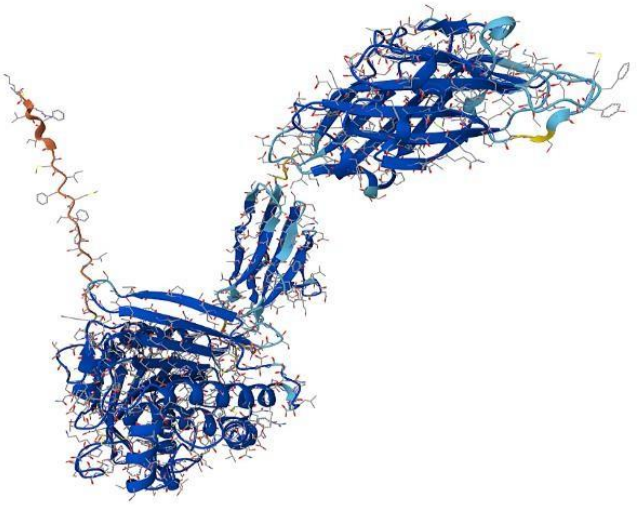


Figure 6. Poly(3-hydroxybutyrate) Depolymerase Protein of *Bacillus subtilis* Encoded from gene *phaZ* (Protein Structure Extracted from AlphaFold Database; https://alphafold.ebi.ac.uk/entry/A0A8W8).

The PHA biosynthetic pathway was studied using KeGG database through butanoate metabolism (Figure 7). It was found that the short chain length (scl-PHA) biosynthetic pathway involves three enzymatic reactions. PhaA condenses two acetyl CoA molecules into one acetoacetyl CoA, which is reduced to R-3-hydroxyacyl CoA monomers by NADPH dependent PhaB. PhaC polymerises these monomers to polyhydroxybutyrate. In case of medium chain length (mcl-PHA), fatty acid biosynthetic pathway is followed via either de novo synthesis or β-oxidation [34, 35]. Following the β-oxidation route, R-3-hyroxyacyl CoA can be produced through the polymerization of trans-2-enoyl CoA, S-3-hydroxyacyl CoA and 3-ketoacyl CoA by hydratases (PhaJ), epimerases, and reductases (PhaB) [36]. Following the de novo synthesis route, R-3-hyroxyacyl CoA is produced through the action of transacylases (PhaG) that polymerises R-3-hyroxyacyl ACP [37]. Gene phaZ, on the other hand, encodes specific intracellular depolymerase of medium chain length that is involved in polymer depolymerization, that is, depolymerization of plastics [38, 39].

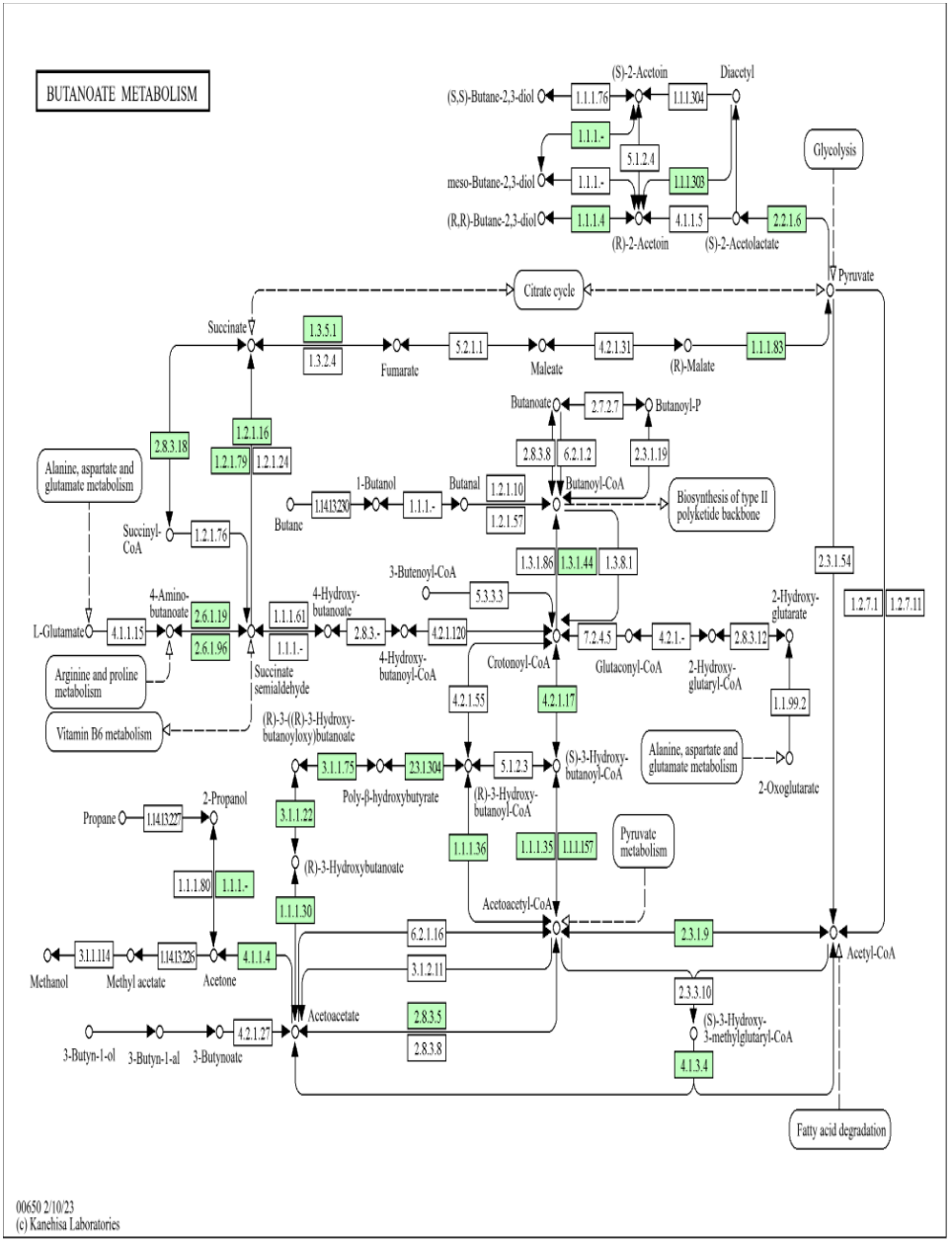


Figure 7. PHA Biosynthesis via Butanoate Metabolism Pathway (Extracted from KeGG)

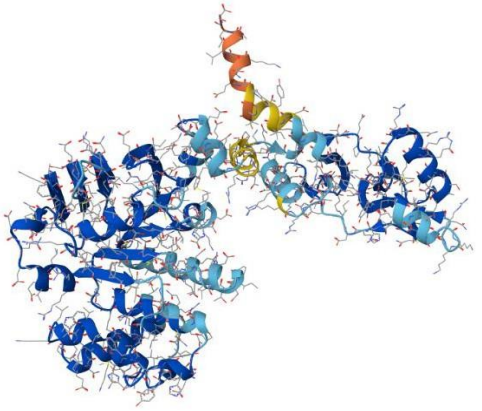


Figure 8. Choline Transport ATP-binding Protein OpuBA of *Bacillus subtilis* Encoded from Gene *opuBA* (Protein Structure Extracted from AlphaFold Database; https://alphafold.ebi.ac.uk/entry/Q45460).

Since PHA production occurs under extreme environmental stress, genes associated with survival mechanisms, such as salt tolerance, were also identified. Protein product of gene *opuBA* is visualized below using AlphaFold (Figure 8). The ABC transporter family containing the *NatAB* gene (Na^+ extrusion transporter) was also found in the *Bacillus subtilis* PWA genome. This *NatAB* system is concerned with electrogenic Na+ extrusion, coupled with ATP dependent uptake. The *OpuD* and *OpuE* genes are associated with Na^+-coupled sodium-solute symporter (SSS) family. The glycine betaine and L-proline transporters simultaneously generate compatible solutes, in turn inducing the need for the quick removal of Na^+ ions. The betaine aldehyde dehydrogenase (*badh*) gene is related to mechanisms dealing with salinity and drought stress. Genes affiliated with heat shock proteins DnaK, DnaJ, ClpXA were also identified in *Bacillus* genome [40].

Overall, the *Bacillus subtilis* PWA exhibited various beneficial traits, such as plastic bioconversion to biopolymers (plastic biodegradation with simultaneous biopolymer biosynthesis) and halotolerance. However, the functional roles of the identified genes were inferred bioinformatically, without experimental validation. Future studies could be aimed at the functional characterization of key genes involved in plastic degradation (e.g., *alkB1-3*, *CYP153*) and PHA biosynthesis (*phaA*, *phaB*, *phaC*, and

phaZ). Furthermore, metabolic engineering of PHA operon could significantly increase PHA yield, monomer diversity, and volumetric productivity.

3.1. Conclusion

The genome analysis of *Bacillus subtilis* PWA showed the presence of various genes involved in plastic degradation, biopolymer production, and mediation of salt tolerance. The presence of PHA biosynthetic gene *phaC* exhibited the ability of the bacterial strain for PHA production, whereas the presence of *alkB* and *phaZ* genes accounted for polymer biodegradation. The explorative analysis of bacterial whole genome also shed light on the PHA biosynthetic pathway. Further in depth studies can aid in tuning the PHA cycle for volumetric biopolymer yield.

Author Contribution

Rafeya Sohail: conceptualization, formal analysis, investigation, methodology, writing-original draft, writing-review & editing. **Rida Batool:** supervision, validation. **Nazia Jamil**: visualization, resources, supervision.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Genomic datasets (raw reads and assembly) are available in public genome repository; NCBI GenBank under assembly number GCA_047314825.1.

Funding Details

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Generative AI Disclosure Statement

The authors did not used any type of generative artificial intelligence software for this research.

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