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An *In-Silico* Approach of Polyhydroxybutyrate Synthesis and Phylogeny Study for Degradation of Polyhydroxybutyrate in Organisms from Lower to Higher Organization

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Abstract: The in-silico approach is common in today's world. As it provide a vast knowledge of hypothetical world which have to be proven by undergoimg in in-vitro conditions. There are much data is available on databases which helps to complete the future study related to medicine, environment and nano-technology. The study covered the new ideas which can able to change the approach of biosynthesis of PHB in microorganisms and degradation of biopolymer without any harmful effect on environment as well as ecosystem.
Keywords: Polyhydroxybutyrate, Phylogeny tree, Polyesters, Biodegradable, Biosynthesis, Rlastonia eutropha

I. INTRODUCTION

In-silico appraoch is necessary to start any project in medical as well as environmental field. As it contains data which is required for many studies. This work is done with the help of online tools and databases. PHB biosynthesis is important to understand the basic need and requirement of microorganisms for their survival in non favourable conditions. PHB is storage material which is synthesised by acety co-A moiteies as their raw material (Luengo *et al.*, 2003). Acetyl co-A undergoes in condensation and produces acetoacety co-A with the help of various enzyme activities. The sole purpose of biosythesis od storage material is, limitation o required macromolecules whih stops the nitrogenous enzyme to synthesis protein and further go for cell division. When microbial enzyme activity stops, microbes start synthesis of polyhydroxyalkanoates in the cell which are polyesters for their survival (Luengo *et al.*, 2003). These polyhydroxyalkanoates are of many types which depends on cell type and their habitate. As polyhydroxyalkanoates contains many type of polyesters but this study was foussed on only one type of polyesters which is polyhudroxybutyrate which is highly synthesised bu *Ralstonia eutrophus* which is a gram negative, non-spore forming bacilli. This study's solely focussed on polyhydroxybutyrate because this polyester showing major ressemblence with single use polymer i.e., polyethylene which is synthetically synthesiised and are not able to degrade arter and after several years (Bhat *et al*., 2020). This single use polymer polluted the area on earth with degrade the quality of environment and ecosystem as many animals and ocean animals are died by eating it(Hayden *et al.,* 2013). These study will hange the future world's appraoh for focussing on degradation as well. These biopolymer are not poisnous for mistakenly eating by animals as well as humans becaue humans contains higher enzymes which are able to degrade these biopolyers in the body and remove out without any gene manipulation.

DATABASES: NCBI, BIOCYC, METACYC, MUSCLE/ CLUSTAL W

TOOLS: Comparative analysis, MEGA X

II. METHODS

A. Selection of Suitable Strain of Microorganism

1) Search the site of NCBI (ncbi.nih.nlm.in).
2) Open the home page of NCBI.
3) Choose the 'all genome' option from left search column.
4) Choose the 'bacterial name' in right search option.
5) Result shows bacterial FASTA sequence.
6) Select the BLAST program.
7) Enter a query sequence or upload a file containing sequence.
8) Select the database to search.

9) Select the algorithm and the parameters of the algorithm for the search.
10) Run the BLAST program.
11) Optimise the similar of Bacterial genome and select the perfect one.

B. Study of Biosynthesis of Polyhydroxy Butyrate/ butyric acid in Ralstonia eutropha by Biocyc

1) Search the online tool: biocyc.org
2) Type 'Polyhydroxybutyrate' on search column display on right side (up) on the page.
3) Choose the option 'Polyhydroxbutanoate biosynthesis (polyhydroxybutyrate biosynthesis)' out from three results.
4) Study the results of reaction with enzymatic pathways.
5) Select the option 'Multiple Database' from right side (down) the page.
6) Collect the data of same reaction in multiple databases.

C. Use of metacyc tool for study of Polyhydroxybutyrate synthesis in Microorganisms

1) Search metacyc.org
2) Enter Polyhydroxybutyrate in search column
3) Click on pathway of Polyhydroxbutanoate biosynthesis (polyhydroxybutyrate biosynthesis).
4) Retrieve the pathway and collect the data
5) Search this pathway in Multiple Database

D. Comparative Analysis for Cupriavidus necator H 16

1) Search the online tool biocyc.org.
2) Enter polyhydroxybutyrate in search column.
3) Click on pathway of Polyhydroxbutanoate biosynthesis (polyhydroxybutyrate biosynthesis).
4) Run the speicies comparison
5) Go on comparative analysis start page option given on last of the page.
6) Select Pathways: breakdown by pathway class, information on pathway holes.
7) Select 'choose organism' for comparative analysis
8) Add microorganisms according to taxonomy
9) Select pathway option and optimize the data

III. PHYLOGENETIC TREE PRODUCTION BY MEGA X SOFTWARE

For alignment

1) Go to "Align (dropdown) **-->** Edit/Build Alignment **-->** Retreive sequences from a file **-->** OK".
2) Selected the input file which was in fasta format. A new window was open showing all the sequences.
3) Go to "Edit **-->** Select All" or simply press Ctrl+A.
4) Go to "Alignment **-->** Align by MUSCLE **-->** Align Protein **-->** OK**"**. This software can align sequences by ClustalW by selecting "Align by ClustalW" instead of selecting "Align by ClustalW" from the *Alignment* option at the top menu bar.
5) After processing, it was showed the aligned sequences in the same window.
6) If wanted then saved the session, then go to "Data **-->** Save Session". Select the appropriate folder and click *Save.*

A. Exporting into the MEGA format

1) Go to Data **-->** Export Alignment **-->** Mega Format. DATA was also export into other formats such as FASTA, Phylip/Paup at this step.
2) Selected the appropriate folder and clicked *Save.*

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B. Constructing the Phylogenetic Tree

1) Go to the main window of MEGAX. Click Phylogeny --> Construct/Test Maximum Likelihood Tree.
2) Select the converted file (.meg) and click *Open.*
3) A new window will appear *'Analysis Parameters'*. Here, set the different values such as bootstrapping value, substitution model, etc., It is recommended to test phylogeny by bootstrapping for 500-1000 times. Additionally, selected the substitution model appropriately.
4) After setting parameters, click *Compute*. It was time taken which depending upon the number of sequences and bootstrap values.
5) Finally, it would showed the constructed tree. Save the tree session and export it into Newick format.

IV. RESULTS AND DISCUSSIONS

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns

select all *0 sequences selected*

Description	Scientific Name	Max Score	Total Score	Query Cover	E value
acetyl-CoA acetyltransferase, cytosolic [Bactrocera dorsalis]	Bactrocera dors...	347	759	75%	7e-171
PREDICTED: acetyl-CoA acetyltransferase, cytosolic [Bactrocera latifrons]	Bactrocera latifr...	360	735	75%	1e-169
acetyl-CoA acetyltransferase, cytosolic [Zeugodacus cucurbitae]	Zeugodacus cuc...	327	686	75%	1e-155
acetyl-CoA acetyltransferase, cytosolic [Bactrocera oleae]	Bactrocera oleae	320	680	72%	1e-154
acetyl-CoA acetyltransferase, cytosolic [Rhagoletis pomonella]	Rhagoletis pom...	311	655	72%	3e-147
PREDICTED: acetyl-CoA acetyltransferase, cytosolic [Rhagoletis zephyria]	Rhagoletis zeph...	310	654	72%	1e-146
unnamed protein product [Ceratitis capitata]	Ceratitis capitata	307	659	75%	7e-146
acetyl-CoA acetyltransferase, cytosolic [Ceratitis capitata]	Ceratitis capitata	300	642	75%	8e-144
acetyl-CoA acetyltransferase, cytosolic [Lucilia cuprina]	Lucilia cuprina	272	618	74%	1e-131
acetyl-CoA acetyltransferase, cytosolic [Lucilia sericata]	Lucilia sericata	273	617	74%	2e-131
hypothetical protein DOY81_003424 [Sarcophaga bullata]	Sarcophaga bull...	272	608	73%	7e-129
PREDICTED: acetyl-CoA acetyltransferase, cytosolic [Musca domestica]	Musca domestica	276	607	75%	2e-128
LOW QUALITY PROTEIN: uncharacterized protein LOC110186976 [Drosophila serrata]	Drosophila serrata	259	568	74%	1e-124
acetyl-CoA acetyltransferase, cytosolic [Teleopsis dalmanni]	Teleopsis dalma...	257	589	75%	3e-124

Result 1: BLAST results of strain selection for PHB synthesis

Ralstonia eutropha H16 was taken for this study because this strain of organism produces polyhydroxybutyrate in large amount than other strain. They are facultative aerobes that synthesize Polyhydroxybutyrate keto-acids in the absence of Oxygen and higher Carbon amount. The role of PHB synthesis is, it produces energy for microbial survival in such conditions. Another major advantage of the selected strain was, it is a non-spore-forming, non-pathogenic gram-negative bacteria.

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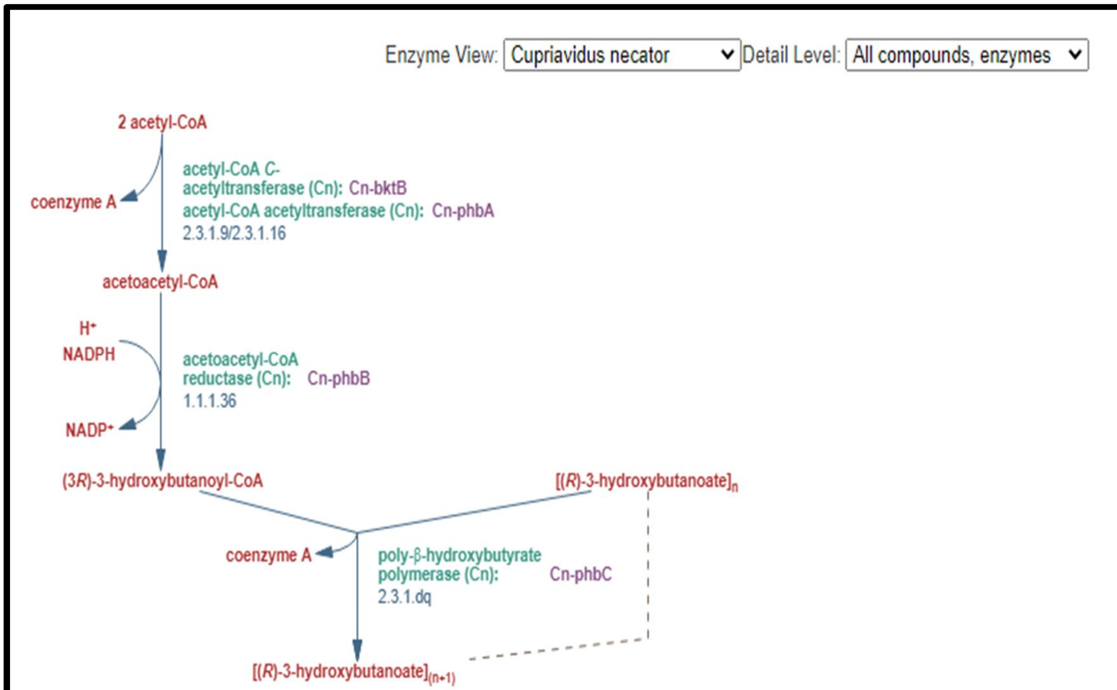
Organism (Database) ▲ ▽	Pathway △ ▽
[Pseudomonas] pictorum JCM 9942	polyhydroxybutanoate biosynthesis
Acetobacteraceae bacterium AT-5844	polyhydroxybutanoate biosynthesis
Acetobacteraceae bacterium AT-5844	polyhydroxybutanoate biosynthesis
Achromobacter denitrificans NBRC 15125	polyhydroxybutanoate biosynthesis
Achromobacter denitrificans USDA-ARS-USMARC-56712	polyhydroxybutanoate biosynthesis
Achromobacter insuavis AXX-A	polyhydroxybutanoate biosynthesis
Achromobacter piechaudii ATCC 43553	polyhydroxybutanoate biosynthesis
Achromobacter ruhlandii SCCH3:ACH 33-1365 (GCF_002082135.1)	polyhydroxybutanoate biosynthesis
Achromobacter sp. DMS1	polyhydroxybutanoate biosynthesis
Achromobacter sp. HMSC070F04	polyhydroxybutanoate biosynthesis
Achromobacter spanius DSM 23806 (GCF_002812705.1)	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans C54	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans HMSC056C09	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans HMSC057D05	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans HMSC15D03	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans HMSC18C08	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans NH44784-1996	polyhydroxybutanoate biosynthesis
Achromobacter xylosoxidans serovar "not known" str. NCTC10807 (GCF_001457475.1)	polyhydroxybutanoate biosynthesis
Acidibrevibacterium fodinaquatile G45-3 (GCF_003352165.1)	polyhydroxybutanoate biosynthesis
Acidiphilium angustum ATCC 35903 (GCF_000701585.1)	polyhydroxybutanoate biosynthesis
Acidiphilium cryptum JF-5	polyhydroxybutanoate biosynthesis
Acidiphilium multivorum AIU301	polyhydroxybutanoate biosynthesis
Acidiphilium sp. PM	polyhydroxybutanoate biosynthesis
Acidisphaera rubrifaciens HS-AP3	polyhydroxybutanoate biosynthesis
Acidocella aminolytica 101 = DSM 11237	polyhydroxybutanoate biosynthesis

Result 2: Pathway in multiple database by BioCyc

Metabolic pathway of any organism shows its whole process of synthesizing and degradation as per requirement of survival. Metabolic pathways the utilisation of macromolecules for further reations. Metabolic pathway for polyhydroxybutyrate synthesis *in-vivo* was observed and studied with BioCyc.



Result 2.1: Biosynthesis of Polyhydroxybutyrate in *Cupriavidus necator* H16 by MetaCyc

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Metacyc online tool provided data of different pathways for multiple reactions at a time which a microbial cell facilitates. Acquired vast knowledge from initial to the final stage. As it cleared all the queries related to Polyhydroxybutyrate synthesis *in-vivo*. For example, acetyl co-enzyme plays the role of substrate for Biosynthesis of PHB with multiple enzyme activities in multiple stages but when and how acetyl co-enzyme undergo for the further reaction of producing PHB *in-vivo*. Synthesis of PHB in microbes complete in 3 steps which occurs in hypoxia condition or facultative microbes undergo fermentation during starvation. These steps are: **Step 1**: Acetyl Co-A synthesized from a different metabolic reaction, undergo the condensation process in which two moieties of acetyl Co-A condense with the utility of 3- Ketothiolase to produce a molecule Acetoacetyl Co-A. **Step 2:** In the second step, Acetoacetyl Co-A reduces by the process of NADPH- dependent Acetoacetyl Co-A reductase to produce (R)- 3- hydroxybutyrate Co-A. **Step 3:** In the last step, PHB synthase synthesis and merge 3 hydroxybutyrate moieties to produce the Poly 3-hydroxybutyrate backbone.

Comparative Analysis Summary Results

Note: In addition to reflecting differences in biology of different organisms, these statistics will reflect differences in the levels of curation, data availability, and completeness of the PGDBs for these organisms.

Comparative analysis and statistics were computed for the following organism databases:

- *Aquabacterium olei NBRC 110486*
- *Aquabacterium parvum B6*
- *Aquabacterium sp. NJ1*
- *Aquincola tertiaricarbonis MIMtkpLc11*
- *Cupriavidus necator H16*
- *Cupriavidus necator H16*
- *Cupriavidus necator N-1*
- *Ideonella sakaiensis 201-F6*
- *Ideonella sp. B508-1*
- *Inhella inkyongensis IMCC1713*
- *Leptothrix cholodnii SP-6*
- *Methylibium petroleiphilum PM1*
- *Methylibium sp. CF059*
- *Methylibium sp. T29-B*
- *Mitsuaria chitosanitabida NBRC 102408*
- *Mitsuaria sp. 7*
- *Paucibacter sp. KCTC 42545 KCTC 42545*
- *Ralstonia insidiosa ATCC 49129*
- *Ralstonia insidiosa FC1138*
- *Ralstonia mannitolilytica MRY14-0246*
- *Ralstonia mannitolilytica SN82F48*
- *Ralstonia pickettii 12D*
- *Ralstonia pickettii 12J*
- *Ralstonia pickettii 5_7_47FAA*
- *Ralstonia solanacearum CFBP2957*
- *Ralstonia solanacearum CMR15*
- *Ralstonia solanacearum EP1*
- *Ralstonia solanacearum FJAT-1458*

Result 3.1: Comparative Analysis Summary Results

Pathway Class	*A. olei* NBRC 110486	*A. parvum* B6	*Aquabacterium sp. NJ1*	*A. tertiaricarbonis* MIMtkpLc11	*C. necator* H16	*C. necator* H16	*C. necator* N-1	*I. sakaiensis* 201-F6	*Ideonella sp. B508-1*	*I. inkyongensis* IMCC1713	*L. cholodnii* SP-6	*M. petroleiphilum* PM1	*Methylibium sp. CF059*	*M. sp. T29-B*	*M. chitosanitabida* NBRC 102408	*M. sp. 7*	*Paucibacter sp. KCTC 42545* KCTC 42545
Biosynthesis	167	160	163	175	194	192	223	164	160	167	173	173	175	118	128	128	163
Amine and Polyamine Biosynthesis	3	4	5	5	5	5	4	6	3	2	2	6	5	2	1	1	5
Amino Acid Biosynthesis	26	25	22	25	30	28	36	24	27	29	27	23	26	19	23	23	27
Aminoacyl-tRNA Charging	2	2	2	2	3	1	3	2	2	3	2	2	2	2	3	2	2
Aromatic Compound Biosynthesis	4	4	3	3	3	4	5	3	3	3	4	4	3	3	3	3	3
Carbohydrate Biosynthesis	14	10	11	16	14	15	16	15	9	8	14	11	17	10	6	7	11
Cell Structure Biosynthesis	6	5	5	5	4	5	6	4	5	5	6	5	5	2	5	3	5
Cofactor, Carrier, and Vitamin Biosynthesis	51	52	55	55	59	56	65	49	53	52	56	61	58	32	36	33	52
Fatty Acid and Lipid Biosynthesis	16	15	16	17	16	17	19	19	18	16	17	17	18	14	12	14	16
Metabolic Regulator Biosynthesis	4	1	3	4	5	5	5	4	1	1	1	3	4	2	2	2	1
Nucleoside and Nucleotide Biosynthesis	15	15	15	14	21	18	20	15	13	16	14	14	16	12	15	16	16
Other Biosynthesis	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Polyprenyl Biosynthesis	3	2	4	2	3	3	5	3	2	2	4	2	2	2	2	1	2
Secondary Metabolite Biosynthesis	4	4	4	7	7	6	7	4	5	6	5	5	5	1	2	5	5
Storage Compound Biosynthesis	2	2	2	2	0	1	1	2	2	1	2	2	1	1	0	0	2
Tetrapyrrole Biosynthesis	3	4	4	6	4	4	4	6	4	4	4	5	4	3	3	2	4

Pathway Class: Biosynthesis - Storage Compound Biosynthesis	*A. olei* NBRC 110486	*A. parvum* B6	*Aquabacterium sp. NJ1*	*A. tertiaricarbonis* MIMtkpLc11	*C. necator* H16	*C. necator* H16	*C. necator* N-1	*I. sakaiensis* 201-F6	*Ideonella sp. B508-1*	*I. inkyongensis* IMCC1713	*L. cholodnii* SP-6	*M. petroleiphilum* PM1	*Methylibium sp. CF059*	*M. sp. T29-B*	*M. chitosanitabida* NBRC 102408	*M. sp. 7*	*Paucibacter sp. KCTC 42545* KCTC 42545	*R. insidiosa* ATCC 49129
cyanophycin metabolism	X	X	X	X			X	X	X	X	X	X					X	
polyhydroxybutanoate biosynthesis	X	X	X	X		X		X	X		X	X	X	X			X	X

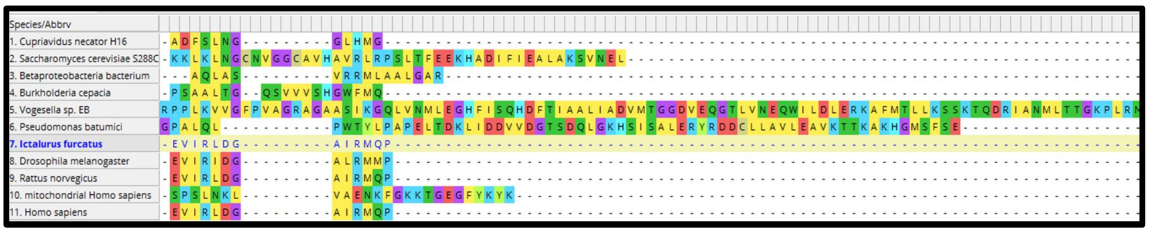
R. insidiosa FC1138	*R. mannitolilytica* MRY14-0246	*R. mannitolilytica* SN82F48	*R. pickettii* 12D	*R. pickettii* 12J	*R. pickettii* 5_7_47FAA	*R. solanacearum* CFBP2957	*R. solanacearum* CMR15	*R. solanacearum* EP1	*R. solanacearum* FJAT-1458	*R. solanacearum* FJAT-91	*R. solanacearum* FQY_4	*R. solanacearum* GMI1000	*R. solanacearum* HA4-1	*R. solanacearum* KACC 10722	*R. solanacearum* KACC10709
X															
X	X	X	X	X	X	X			X			X			X

R. solanacearum MolK2	*R. solanacearum* OE1-1	*R. solanacearum* PSI07	*R. solanacearum* Po82	*R. solanacearum* SL3022	*R. solanacearum* T12	*R. solanacearum* T25	*R. solanacearum* UW386	*R. solanacearum* UW551	*R. solanacearum* YC40-M	*R. sp. 5_2_56FAA*	*R. gummiphilus* NS21	*Rhizobacter sp. Root1221*	*Rhizobacter sp. Root29*	*Rhizobacter sp. Root404*
												X		
			X						X	X		X	X	X

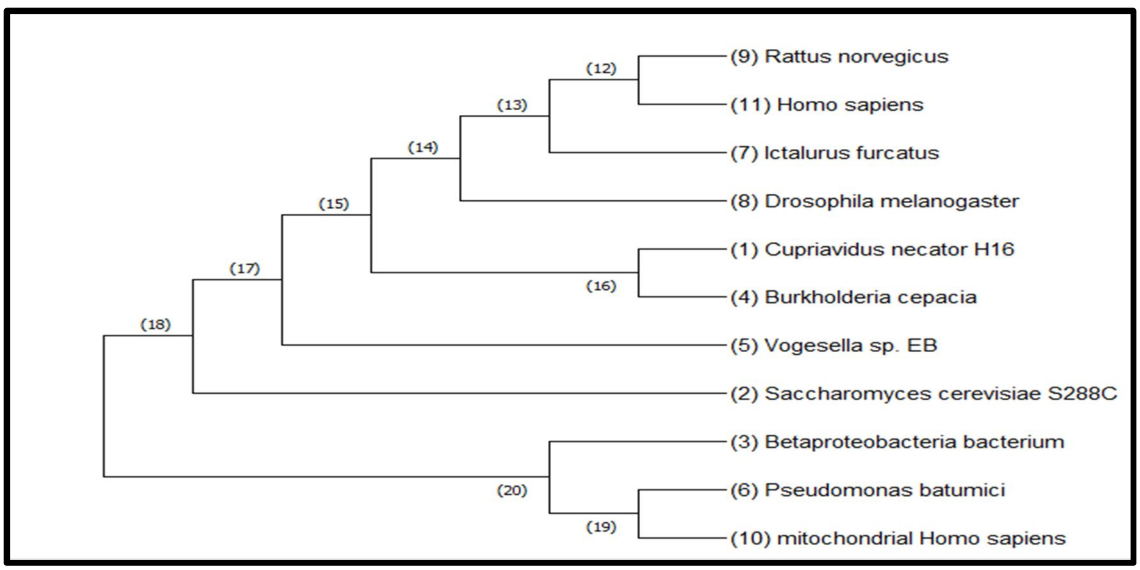
Rhizobacter sp. Root29	*Rhizobacter sp. Root404*	*R. depolymerans* KCTC 42856	*R. benzoatilyticus* JA2	*R. gelatinosus* IL144	*S. natans natans* DSM 6575	*S. natans subsp. sulfidivorans*	*T. fonticaldi* PL17	*T. taiwanensis* VT154-175	*T. arsenitoxydans* 3As	*T. bhubaneswarensis* DSM 18181	*T. intermedia* ATCC 15466	*T. intermedia* K12	*T. sp.* FB-6	*T. sp.* FB-Cd	*X. ampelinus* CCH5-B3	*Xylophilus sp.* Leaf220	*P. brachysporum* DSM 7029
		X		X	X	X		X								X	X
X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X

Result 3.2: Outcomes of Storage Compound Biosynthesis

The major aim of comparative analysis is to identify similarities and differences between different species/taxonomy. Investigation of bacterial communities and diversity is very important as these microbes exert direct beneficial or pathogenic effects on other species. Comparison of the culturable and non-culturable community will help to determine the structurally abundant, functionally viable, and potentially valuable bacteria that can ultimately be used as inoculum for the desired product. This studied was required to check whether the strain selected for study is suitable or not. This study concluded that there are many taxonomy and species which are available for higher productivity nonetheless productive more than *Ralstonia eutropha.*



Result 4.1: Aligned Hydroxybutyrate dehydrogenase Enzyme of different species



Result 4.2: Evolutionary Analysis by Maximum Likelihood Method

The roots of a phylogenetic tree represent the common ancestor of the sequences. Some trees are unrooted, and thus do not specify the common ancestor. A tree can be rooted using an outgroup (that is, a taxon known to be distantly related from all other Operational taxonomic units). Bootstrapping is a statistical technique that tests the sampling errors of a phylogenetic tree. It does so by repeatedly sampling trees through slightly perturbed datasets.

V. CONCLUSION

Ralstonia eutropha H16 was taken for this study because this strain of organism produces polyhydroxybutyrate in large amount than other strain. They are facultative aerobes that synthesize Polyhydroxybutyrate keto-acids in the absence of Oxygen and higher Carbon amount. The role of PHB synthesis is, it produces energy for microbial survival in such conditions. Another major advantage of the selected strain was, it is a non-spore-forming, non-pathogenic gram-negative bacteria. Metabolic pathway of any organism shows its whole process of synthesizing and degradation as per requirement of survival. Metabolic pathways the utilisation of macromolecules for further reations. Metabolic pathway for polyhydroxybutyrate synthesis *in-vivo* was observed and studied with BioCyc. Metacyc online tool provided data of different pathways for multiple reactions at a time which a microbial cell facilitates. Acquired vast knowledge from initial to the final stage. As it cleared all the queries related to Polyhydroxybutyrate synthesis *in-vivo.* For example, acetyl co-enzyme plays the role of substrate for Biosynthesis of PHB with multiple enzyme activities in multiple stages but when and how acetyl co-enzyme undergo for the further reaction of producing PHB *in-vivo.* The major aim of comparative analysis is to identify similarities and differences between different species/taxonomy. Investigation of bacterial communities and diversity is very important as these microbes exert direct beneficial or pathogenic effects on other species. Comparison of the culturable and non-culturable community will help to determine the structurally abundant, functionally viable, and potentially valuable bacteria that can ultimately be used as inoculum for the desired product. This studied was required to check whether the strain selected for study is suitable or not. This study concluded that there are many taxonomy and species which are available for higher productivity nonetheless productive more than *Ralstonia eutropha.* The roots of a phylogenetic tree represent the common ancestor of the sequences. Some trees are unrooted, and thus do not specify the common ancestor. A tree can be rooted using an outgroup (that is, a taxon known to be distantly related from all other Operational taxonomic units). Bootstrapping is a statistical technique that tests the sampling errors of a phylogenetic tree. It does so by repeatedly sampling trees through slightly perturbed datasets. Data were collected from NCBI for producing a phylogeny tree. Each enzyme (Protein) was selected from different species. Collected data is in FASTA sequence form. For MegaX, a sheet was generated and uploaded according to the MegaX sheet format. Sequence after upload was sequence aligned with the help of Muscle/ ClustalW. After all these steps data sheet was prepared for phylogeny tree analysis for evolutionary.

The phylogeny tree was constructed in between enzymes that present in multiple organisms from microbial species to higher eukaryotes. That enzyme was responsible for the synthesis of keto-acids (hydroxybutyrate). Results were showed that positively define the evolution of genes responsible for an enzyme present in almost all organisms. For example, homo sapiens’ liver cells also produce hydroxybutyrate in starvation conditions.

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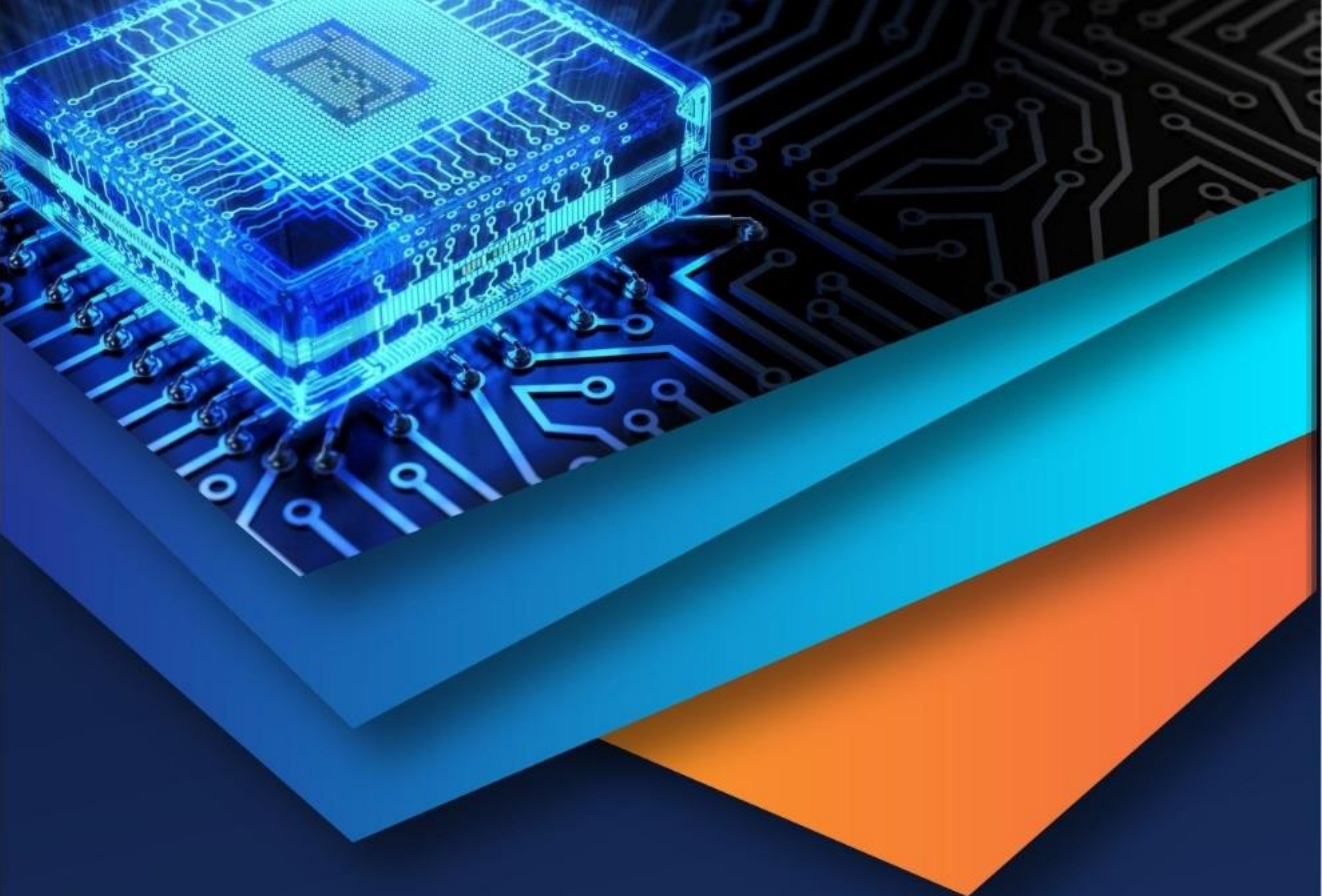

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