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 undergoing 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, Ralstonia eutropha

I. INTRODUCTION

*In-silico* approach 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 acetyl co-A moieties as their raw material (Luengo *et al.*, 2003). Acetyl co-A undergoes in condensation and produce acetoacetyl co-A with the help of various enzyme activities. The sole purpose of biosynthesis of storage material is, limitation of required macromolecules which 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 habitatte. As polyhydroxyalkanoates contains many type of polyesters but this study was focussed on only one type of polyesters which is polyhydroxybutyrate which is highly synthesised by *Ralstonia eutropha* which is a gram negative, non-spore forming bacilli. This study’s solely focussed on polyhydroxybutyrate because this polyester showing major ressemblance with single use polymer i.e., polyethylene which is synthetically synthesised and are not able to degrade after 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 approach for focussing on degradation as well. These biopolymer are not poisnous for mistakenly eating by animals as well as humans because humans contain higher enzymes which are able to degrade these biopolymers 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 species 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.
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

**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 reactions. Metabolic pathway for polyhydroxybutyrate synthesis *in-vivo* was observed and studied with BioCyc.

**Result 2: Pathway in multiple database by BioCyc**

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

**Result 3.1: Comparative Analysis Summary Results**
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 study concluded that there are many taxonomy and species which are available for higher productivity nonetheless productive more than *Ralstonia eutropha*.

**Result 3.2: Outcomes of Storage Compound Biosynthesis**

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.

**Result 4.1: Aligned Hydroxybutyrate dehydrogenase Enzyme of different species**

**Result 4.2: Evolutionary Analysis by Maximum Likelihood Method**
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 reactions. 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 study 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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