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Flux balance analysis for maximizing biomass, *in vitro* production and characterisation of Polyhydroxy butyrate from *Bacillus subtilis and cereus*

Puniethaa Prabhu*, Sangeetha G R, Gowtham Raj M, Sowmiyamathi S, Raviraagul S

[*]Department of Biotechnology, K.S. Rangasamy College of Technology, Tiruchengode, Namakkal, Tamil Nadu, India.

[*]Corresponding author – spunitha156@gmail.com

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ABSTRACT

BACKGROUNDS: A method based on constraints is called Flux Balance Analysis (FBA), it is used to examine metabolic networks, analyse the flow of metabolites in a network and predict the biomass. Polyhydroxy butyrate (PHB) is a type of polyhydroxy alkanoates, a bio-derived plastic with a wide range of applications in packaging films and as a wrapping material. *Bacillus spp.*, a Gram-positive bacterium, is capable of PHB biosynthesis.

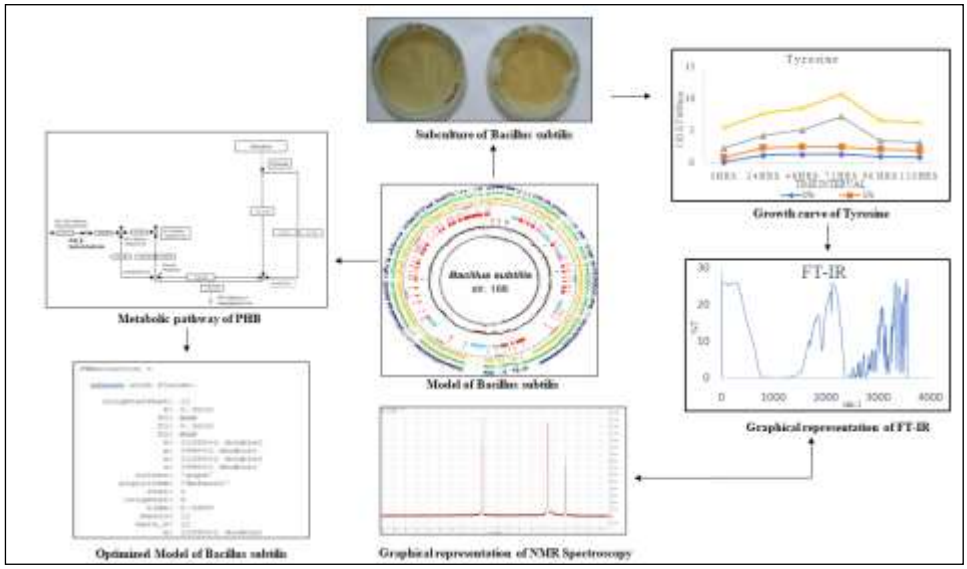
METHODS: The proposed work aimed at understanding the behaviours of *Bacillus spp.*, for the synthesis of PHB with the help of a metabolic flux model using a constraint-based FBA approach. To illustrate the synthesis of PHB in *Bacillus spp.*, the core metabolic network is constructed using the available biochemical data. To assess the flow of metabolites, *in-silico* FBA analysis is used. By changing the upper bound (UB) and lower bound (LB) values and adding additional reactions to boost biomass production, the flux under the steady-state condition is computed and the goal function is established.

RESULTS: The objective function's value is maximised or minimised in the best linear programming (LP) solution. By contrasting it with the experimental data on the rise in biomass production, the model's structural validity was shown. Tyrosine was employed in the *in-vitro* investigations as an amino acid source to stimulate PHB production. The intracellular PHB was extracted using the chloroform-sodium hypochlorite extraction procedure, and Sudan Black B staining was employed as the presumed test for the presence of PHB.

CONCLUSIONS: The experiment produced 0.9 g/ml of residual biomass and 50% of PHB, which is compatible with the theoretical conclusions of the FBA analysis of production potential with tyrosine as an amino acid supply. PHB is more advantageous than polyethylene and polypropylene because it is much less porous, making it a superior material for food packaging without the need for an antioxidant.

Keywords : Flux balance analysis, *Bacillus spp.*, polyhydroxy butyrate, biomass, COBRA toolbox, objective function, and linear programming.

Graphical Abstract



1. INTRODUCTION

Plastics have been an integral part of our lives. Biodegradable plastics, also known as green plastics, are plastics made from biodegradable polymer materials. Polyhydroxy butyrate (PHB) is a suitable source for biodegradable polymer material because of its full degradability and non-pollutant characteristics. Polymers are made up of non-renewable as well as renewable feedstock. These polymers are well known for their diverse applications in industries, domestic appliances, transportation, construction, shelters, storage and packaging practices. Such polymers are differentiated according to their chemical nature, structural arrangement, physical properties and applications. Over the past few years, owing to rising petroleum prices and many environmental concerns related to non-degradable material pollution which have been developed rapidly (Magda M. Aly *et al*., 2019). Polyhydroxy butyrate (PHB) is a bioplastic produced by various microbial sources (Wei *et al*., 2011). In the stationary phase of growth, when the cells are deficient in a necessary nutrient but have an abundance of carbon and amino acid sources, all bacteria capable of producing PHB and it is the first member of the Polyhydroxy alkanoate family (Shah *et al*., 2014). It serves as an energy source during nutrient depletion for bacterial survival. In bacteria, PHB is synthesized from acetyl-CoA in three sequential steps (Gayathiri *et al*., 2017).

- 3-keto thiolase condenses the formation of two acetyl-CoA (acetoacetyl-CoA).
- 2-acetyl-CoA is reduced to R-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase.
- R-3-hydroxy butyryl-CoA is polymerized to form PHB by the catalytic activity of PHB synthase.

PHB (Polyhydroxy butyrate) has emerged as a highly promising alternative to petrochemical plastics and a leading candidate for biodegradable plastics. Its biocompatibility, biodegradability, and versatility make it an excellent eco-friendly substitute for synthetic polymers in various applications. However, the commercial adoption of PHB has been hindered by two primary challenges: poor thermal stability and significant brittleness after prolonged storage. These limitations have affected its suitability for certain industrial uses and have limited its overall commercial utility. Researchers and scientists continue to explore strategies to address these issues and enhance the practicality of PHB as a sustainable plastic alternative (Neha Sharma, 2019 ; Shah K R, 2014).

Dynamic Flux Balance Analysis (DFBA) is a valuable method employed to investigate the impact of the extracellular environment on microbial metabolism, enabling predictions and optimizations. Through case studies, DFBA demonstrates several essential requirements, such as incorporating individual species' metabolic reconstructions, formulating extracellular mass balances, identifying substrate uptake kinetics, numerically solving coupled linear program/differential equations, and adapting models for common growth conditions and species interactions. However, these challenges can be overcome through computer simulations, which offer a powerful means to identify highly promising conditions. In the realm of modeling and simulation, two commonly used approaches are Ordinary Differential Equations (ODE) and flux balance analysis (FBA). It operates on the principle of mass conservation within a network and enables the analysis of metabolite flows. FBA is particularly employed to predict the growth rates of organisms (Henson *et al*., 2014).

2. MATERIALS AND METHODS

2.1 METABOLIC MODEL

The genome-scale metabolic model of *Bacillus spp.,* titled iYO844 was downloaded in MAT format from the biochemical network reconstruction database maintained by BIGGMODELS (http://bigg.ucsd.edu/models/iYO844). The last version of the iYO844 model contained 990 metabolites, 1250 metabolic reactions, and 844 genes. Biomass growth is the objective function for iYO844.The bounds of the required reaction are changed using the 'changeRxnBounds' function in COBRA Toolbox. The specific sources uptake rate for *Bacillus subtilis* 168 in minimal media was set to 100 mmol gDW-1h-1 according to the value experimentally obtained for *Bacillus subtilis* fermentation. The model is then primed for further analysis including flux balance analysis. The data for the network construction were retrieved from various network databases: BIGG Models, GeneDB, KEGG (Kyoto Encyclopedia of Genes and Genomes), MetaCyc and BRENDA. Some data were retrieved from (F. Pardhelha *et al.*,2013). Orth *et al.,* (2013), performed the biochemical network reconstruction. Their work provides an introduction to metabolic and regulatory network reconstruction and gave a complete description of the core *E. coli* metabolic model which is analyzed in computational format; MATLAB.The biochemical network can be represented as a stoichiometric-based set of equations. At steady state, the dot product of the matrix having flux by v, of length n, and the concentration of the metabolite by vector X, of length m, is given by: The stoichiometry matrix

$$S(m*n)$$

was constructed where m is the number of metabolites and n is number of reactions. In steady state

$$S.v = 0$$

where v is the flux of reactions (Kauffman et al. 2003). Objective function is

$$Z = w.v$$

where 'w' is the weight vector of the reactions. Objective function (Z) can be maximized (ZMAX) or minimized (ZMIN)

$$S.v = 0$$

The complete list of reactions, metabolites, and metabolic reactions involved. The data for the network construction were retrieved from various network databases: Gene DB, KEGG, MetaCyc, and BRENDA. Some data retrieved from K.E. JTyo *et al*.,2010; F. Pardhelha*et al*.,2013;M. Lopar*et al*., 2013. The biochemical model of *Bacillus subtilis*, iYO844 was constructed based on the genomic data. The core metabolic model of *Bacillus subtilis* was studied for flux balance analysis of the synthesis of Polyhydroxy butyrate. For instance, employing such models to make personalised predictions can result in a precise phenotypic characterisation of patients (Nisha Sanjay Barge *et a*l., 2023).

2.2 COBRA TOOLBOX

The constraint-based analysis and reconstruction (COBRA) MATLAB® toolbox is available for free download at http://systemsbiology.ucsd.edu/Downloads/Cobra_Toolbox. A wide range of metabolic characteristics are frequently modelled, examined and forecasted using genome-scale biochemical

networks. In COBRA, models are represented by fields with names like as "mets" (a list of all metabolite names), "rxns" (reactions), and "S" (the stoichiometric matrix). Use the optimizeCbModel function to carry out FBA. To modify the reaction limits, use the function "changeRxnBounds" (Ng *et al*., 2018).

2.3 CONSTRAIN BASED MODELLING AND FLUX BALANCE ANALYSIS

The most popular constraint-based method for predicting flux distributions and network capabilities in genome-scale models is Flux Balance Analysis (FBA). This makes it ideal for metabolic engineering experiments that pinpoint and characterise the best perturbations, such as various substrates or genetic alterations that compel a link between growth rate and excess production of the desired metabolite. FBA is a strong technique for predicting how cells behave under various metabolic situations (Nisha Sanjay Barge, 2023).

2.4 FLUX BALANCE ANALYSIS

2.4.1. Optimizing the model

FBA is based on the linear optimization of an objective function, which typically is biomass formation. Two functions in the COBRA Toolbox solve this problem and display the results. FBASolution = optimizeCbModel (model, osenseStr, minNorm, allowLoops, zeroNormApprox). This function performs FBA on the model in order to maximize the current objective function. The result is the data structure solution, which contains an optimal solution for the model that was input. The field ‘‘x’’ describes a particular, possibly non-unique, optimal flux distribution through the network. The field ‘‘f’’ gives the objective value corresponding to the predicted unique optimal growth rate.

FBAsolution.obj > 0 - The model can produce a biomass component, and proceed with the next biomass component.

FBAsolution.obj = 0 - The model cannot produce biomass component.

2.4.2. Metabolic Pathway

A metabolic pathway designs for PHB production from *Bacillus Subtilis* (iYO844 model) Figure 1 based on FBA the reactions in these pathways are involved in the synthesis of PHB productions these reactions are based on the KEGG databases.

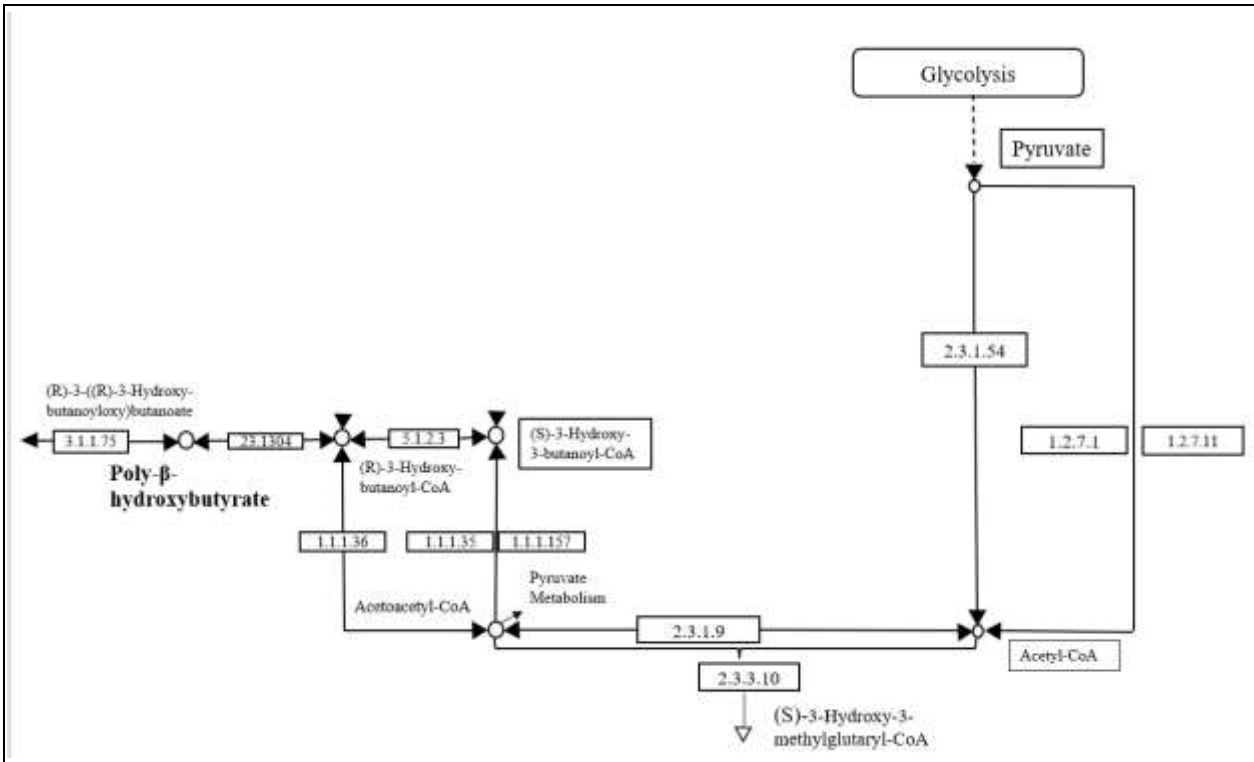


Figure 1. Metabolic Pathway designs for PHB production from *Bacillus Subtilis* (iYO844 model) based on FBA

2.5 COLLECTION OF BACTERIALSAMPLES

The pure culture of *Bacillus spp.,* was collected from MTCC1133 and sub-cultured. It was grown in nutrient broth at 37°C for 24 hours under shaking conditions.

2.5.1 OPTIMIZATION OF PHB PRODUCTION

The *Bacillus spp.,*was inoculated in a minimal medium of different carbon, nitrogen, phosphorous and amino acid sources, with varying concentrations.

2.5.2 PHB PRODUCTION BY *BACILLUS Spp.,*

All the bacterial species are capable for the synthesis of PHB. The *Bacillus spp.,* is grown in a mineral medium with various sources.200ml of Minimal medium supplemented with sources (20g/l).The flasks were maintained at 30°c for 5 days on a rotary shaker at 150 rpm (Hasan M A *et al*., 2018). Metabolic flux analysis is the method to evaluate the number of intracellular metabolites flowing in the central carbon metabolism from knowledge of the rate of production of extracellular products, the rate of substrate utilisation, the rate of product formation, and the rate of biomass production (Ramalingam *et al*., 2010).

2.5.3 EXTRACTION AND QUANTIFICATION OF PHB

A 3 ml of bacterial culture grown in minimal media was transferred to a centrifuge tube and centrifuged at 5000 rpm for 10 min. The obtained pellets were washed with acetone and ethanol (1:1 v/v) and resuspended in a 4 percent sodium hypochlorite solution; incubated at 37 °C for 30 minutes; and centrifuged. The polymer was then dissolved in hot chloroform and kept for complete evaporation. It was then dried, and the dry weight of PHB was calculated. Residual Biomass is calculated as the difference between the cell's dry weight and the dry weight of PHB. (Mukesh *et al*., 2016), studied the PHB production employing eco-friendly and renewable agro products. The extracted PHB granules were analysed by FTIR (Fourier-transform infrared spectroscopy) and Nuclear MagneticResonanceQuantification was done by using the following formula:

Residual Biomass(g/ml) = Dry cell weight – Dry weight of PHB

$$\text{PHB accumulation (\%)} = \left(\frac{Dry\ weight\ of\ PHB}{Dry\ cell\ weight}\right)*100$$

2.6 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR)

Fourier-transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of solid, liquid or gas by absorption or emission. FTIR Analysis or FTIR Spectroscopy is an analytical technique to identify organic, polymeric, and inorganic materials in the given sample. The FTIR analysis method uses infrared light to scan test samples and observe chemical properties. FTIR spectroscopy in combination with the partial least squares (PLS) multiple statistical technique was used for quantitative analysis of the poly (β-hydroxy butyrate) (PHB) contents of the bacterial cells.

2.7 NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a strong constant magnetic field are perturbed by a weak oscillating magnetic field (in the near field) and respond by producing an electromagnetic signal with a frequency characteristic of the magnetic field at the nucleus. NMR can be used to analyse various polymers, including poly hydroxy butyrate (PHB). PHB is a biodegradable polyester that is synthesized by microorganisms and is a potential substitute for petroleum-based plastics (Mikilli *et al*., 2014).

3. RESULT AND DISCUSSION

3.1 Analysis of *Bacillus subtilis* iYO844 model

The core metabolic model, iYO844, was constructed based on the data collected from genome databases and literature sources. The "iYO844.mat" file was loaded into the MATLAB workspace. The model was optimized using the COBRA Toolbox. The model was reconstructed by adding the reactions for PHB synthesis. PHB synthesis reactions were added to the model. KEGG databases are used for the collection of reactions that are involved in the Biosynthesis pathway of PHB.

Reaction 1: β-ketothiolase

2 Acetyl-CoA ↔ Acetoacetyl-CoA + CoA

Reaction 2: Acetoacetyl-CoA reductase

Acetoacetyl-CoA + NADPH + H^+↔ (R)-3-hydroxybutyryl-CoA + $NADP^+$

Reaction 3: PHB polymerase

n (R)-3-hydroxybutyryl-CoA → Polyhydroxy butyrate + n CoA

The "iYO844.mat" mat file consists of reactions, metabolites, a stoichiometric matrix, constraints, reaction names, a metabolite name, and a metabolite formula. The optimized model is given with a result of FBA.obj = 0.1180. The results showed that the model evaluation had a solution status of 1, so it can be concluded that the model is optimized. The model iYO844 was modified by constraining the flux bounds of tyrosine exchange. The results showed that the increase in biomass increased the PHB rate. The optimized reconstructed model gave FBA.obj = 0.3010, which was greater than zero. The reaction could proceed to the next compartment for PHB synthesis.

3.1.1 Production Potential with Sources

The availability of carbon sources is essential for Biomass production. An increase in carbon flux increases Biomass production. The core model was analysed and the carbon fluxes were optimized for an increase in Biomass production.

EX_sub = {"EX_glc__D_e" "EX_sucr_e"}

The core model was analysed, and the amino acid fluxes were optimized for increased biomass production.

EX_AA = {"EX_tyr___L_e"}

The core model was analysed, and the phosphorous fluxes were optimized for increased biomass production.

EX_P ={'EX_pi(c)'}

The core model was analysed, and the nitrogen fluxes were optimized for an increase in biomass production.

EX_N ={'EX_HACD2'}

Table 1. *In-silico* growth of *Bacillus subtilis* using different various sources

Sources	Constraints (mmol/gDW-h)		*fval*
Glucose	UB	100	0.2053
	LB	-1.4	
Sucrose	UB	300	0.2018
	LB	-1	
Tyrosine	UB	100	**0.3010**
	LB	-15.5	
Ammonium Phosphate	UB	500	0.1170
	LB	1	
Potassium Phosphate	UB	300	0.1181
	LB	-1	

Table 1 displays the optimization results of the model after incorporating various sources, including Glucose, Sucrose, Tyrosine, Ammonium Phosphate, and Potassium Phosphate, under different constraints. Among these sources, the Amino Acid Source of the Tyrosine compound exhibits the highest value of FBA.obj (fval) = 0.3010 when the upper bound (UB) constraint is set to 100 mmol/gDW-1 and the lower bound (LB) constraint is set to -15.5 mmol/gDW-1. This indicates that the Tyrosine compound as an amino acid source has the greatest impact on the model's optimization, resulting in the highest objective function value.

3.2 Screening of PHB

After 96 hrs of incubation, the culture was smeared on a glass slide; heat fixed and stained using Sudan Black B. The smear was observed under a light microscope at 100X magnification. The staining with Sudan Black B showed blue-black colour. More PHB granules were spotted as the concentration of carbon sources increases. During microscopic visualization, PHB granules appeared as black granules with Sudan Black B stain.

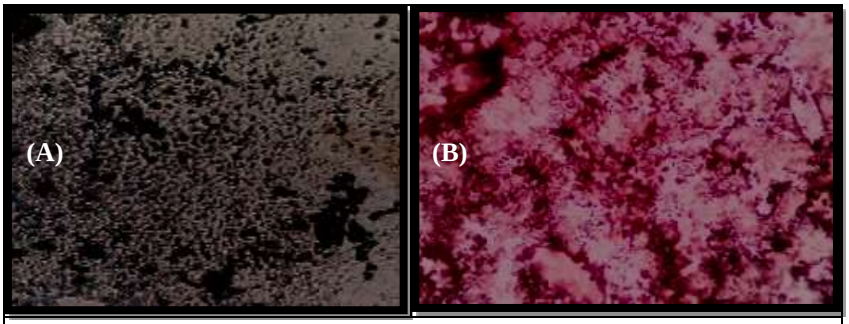


Figure 2: Microscopic examination of Sudan black B screening using (A) Sucrose (B) Glucose

Figure 2 (A) shows the PHB granules during Sudan Black B screening of bacterium grown in 1 % Sucrose. Figure 2 (B) shows the PHB granules during the Sudan Black B screening of bacterium grown in 1 % glucose of Bacillus spp.,

3.2.1 Growth Curve Studies

The *Bacillus spp.*, was sub-cultured in nutrient agar and incubated at 37°C for 24 hrs. The effect of various sources on PHB production was determined by raising the cultures in 100 ml of minimal salt medium in different concentrations of various sources. The cultures were incubated and quantified spectrophotometrically. The growth curve was plotted against obtained OD at 600 nm and time interval.

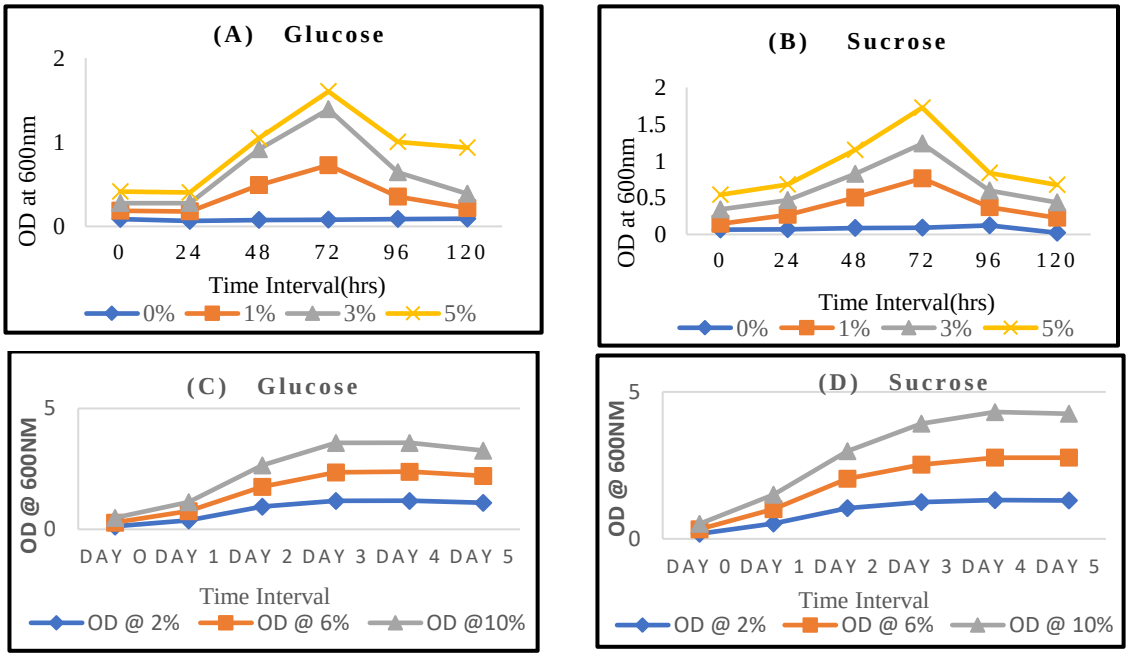


Figure 3. Growth curve of Bacillus spp., in different concentrations of carbon sources for *Bacillus Subtilis* (A) Glucose (B) Sucrose *Bacillus cereus* (C) Glucose (D) Sucrose

In Figures 3 (A) and (B) above, the relationship between the concentration of different carbon sources, such as glucose and sucrose, and the growth of the bacterium is depicted. It is observed that as the concentration of these carbon sources increases, the growth of the bacterium also increases. For glucose, the intensity of growth is highest in media supplemented with 3% glucose, resulting in an optical density (OD) of approximately 0.66 nm. Similarly, for sucrose, the intensity of growth reaches its peak in media supplemented with 5% sucrose, resulting in an OD of about 0.49 nm for *Bacillus subtilis*. Additionally, in the case of *Bacillus cereus*, Figure 3 (C) demonstrates that the intensity of

growth is highest in media supplemented with 10 % glucose, resulting in an OD of about 3.5 nm. Similarly, figure 3 (D) shows that media supplemented with 10 % sucrose yields an OD at about 4.3 nm, indicating the highest intensity of growth among the mentioned OD values for *Bacillus cereus*.

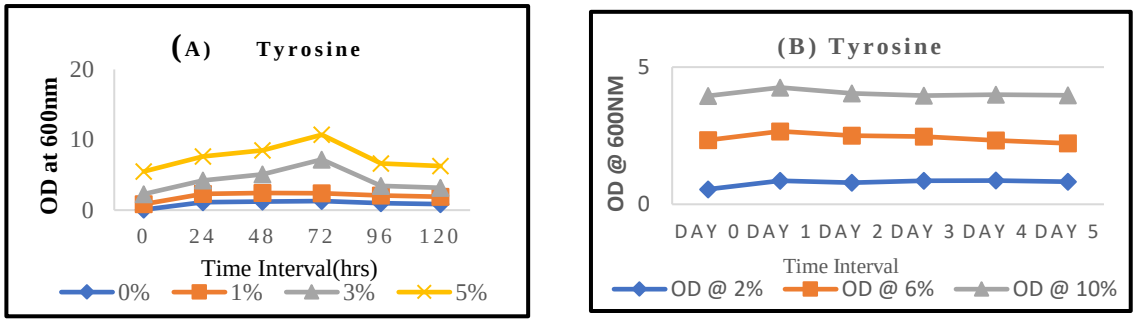


Figure 4. Growth curve of Bacillus spp., in different concentration of tyrosine as amino acid source (A) *Bacillus subtilis* - Tyrosine (B) *Bacillus cereus* - Tyrosine

The above Figure 4 (A) illustrates as the concentration of tyrosine as amino acid source increases, the growth of the bacterium has also increased. In tyrosine intensity of growth was high in media supplemented with 5% Tyrosine has an OD of about 3.51nm in *Bacillus subtilis.* Among those OD values the intensity of growth was high in media supplemented with 10 % tyrosine has an OD of about 4.2 nm of *Bacillus cereus* Figure 4 (B).

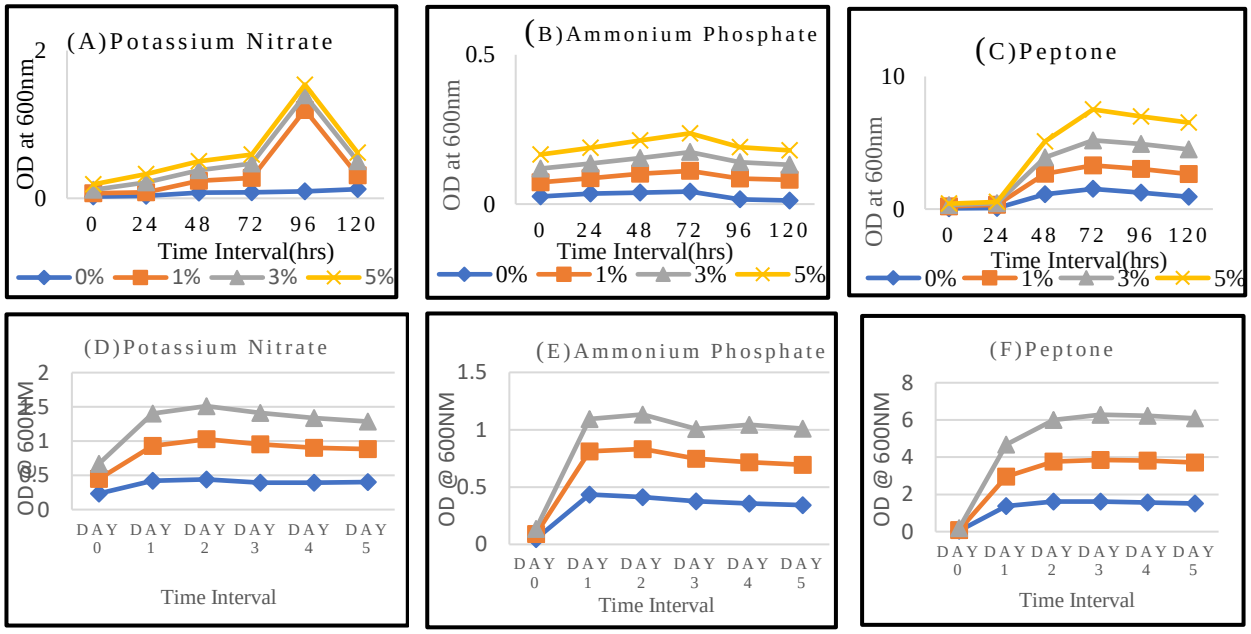


Figure 5. Growth curve in different concentrations of Nitrogen sources, *Bacillus subtilis* (A) Potassium Nitrate (B) Ammonium Phosphate (C) Peptone & *Bacillus cereus* (D), (E), (F)

The above Figure 5 illustrates, as the concentration of various compounds such as Potassium Nitrate, Ammonium Phosphate and Peptone as nitrogen sources increases, the growth of the bacterium has also increased. In Potassium nitrate intensity of growth was high in media supplemented with 3% Potassium Nitrate has an OD of about 0. 19 nm.In ammonium phosphate intensity of growth was high in media supplemented with 5% Ammonium Phosphate has an OD of about 0.063nm. In peptone intensity of growth was high in media supplemented with 5% Peptone has an OD of about 2.32nm in *Bacillus subtilis*and in *Bacillus cereus* of Figure 5 (D) with 10 % ammonium phosphate has an OD of about

1.1nm, figure 5 (E) with 10 % peptone has an OD of about 6.1nm and figure 5 (F) with 10 % potassium nitrate has an OD of about 1.5nm.

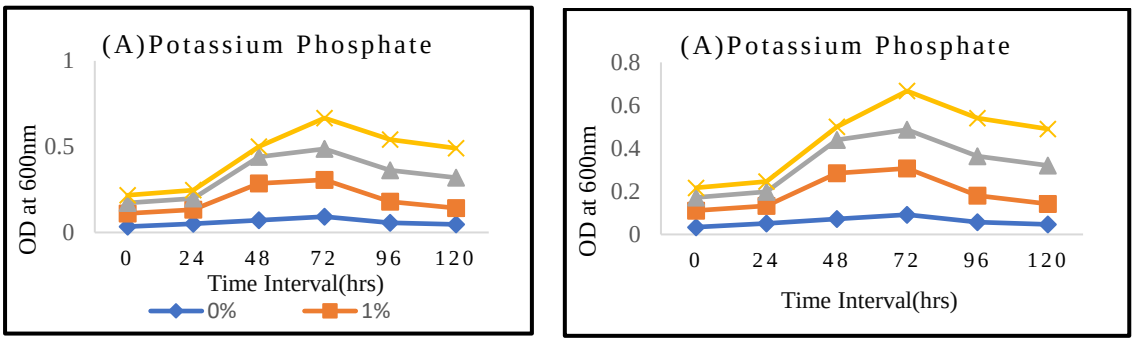


Figure 6. (A) Growth curve of *Bacillus subtilis* and (B) *Bacillus cereus* in different concentrations of Potassium phosphate as Phosphorus sources.

The Figure 6 (A) illustrates, as the concentration of Potassium phosphate as Phosphorous source increases, the growth of the bacterium has also increased. In potassium phosphate intensity of growth was high in media supplemented with 3% Potassium Phosphate has an OD of about 0.183 nm. Among these sources tyrosine has higher growth of OD value of about 3.51nm *in Bacillus subtilis* and Figure 6 (B) with 10 % potassium phosphate has an OD of about 3.6 nm in *Bacillus cereus.*

3.2.2 Biomass of PHB Production

Table 2 shows the result of Dry Cell weight and the Dry weight of PHB obtained from the *Bacillus subtilis* by adding various sources such as carbon, amino acid, nitrogen and phosphorous as its substrate to increase biomass production. Among these, tyrosine as an amino acid source has higher

Table 2. Production of PHB from *Bacillus subtilis* in media with different carbon, amino acid, nitrogen and Phosphorus sources.

PHB production of 0.9g/l from *Bacillus subtilis* and from *Bacillus cereus* also the production of PHB

	Bacillus Subtilis		***Bacillus cereus***	
	DCW(g/l)	**DPHB(g/l)**	**DCW(g/l)**	**DPHB(g/l)**
Carbon Sources				
Glucose	1.14	0.14	0.99	14.65
Sucrose	0.52	0.07	0.37	19.56
Amino Acid				
Tyrosine	1.8	0.9	0.46	61.34
Nitrogen Sources				
Potassium Nitrate	1.4	0.08	1.31	5.07
Peptone	0.52	0.02	0.56	1.7
Ammonium Phosphate	0.16	0.05	0.14	36.36
Phosphorous				
Potassium Phosphate	1.23	0.09	1.19	8.33

is higher in tyrosine of amino acid sources at 61.34g/l.

4. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FT-IR analysis was carried out for the sample of PHB which produced by 3g of Tyrosine. Perkinelmer manufacturer of ATR FTIR SPECTRUM 100 was used to analysis the sample. FTIR peak visualization was represent in Figure 8.

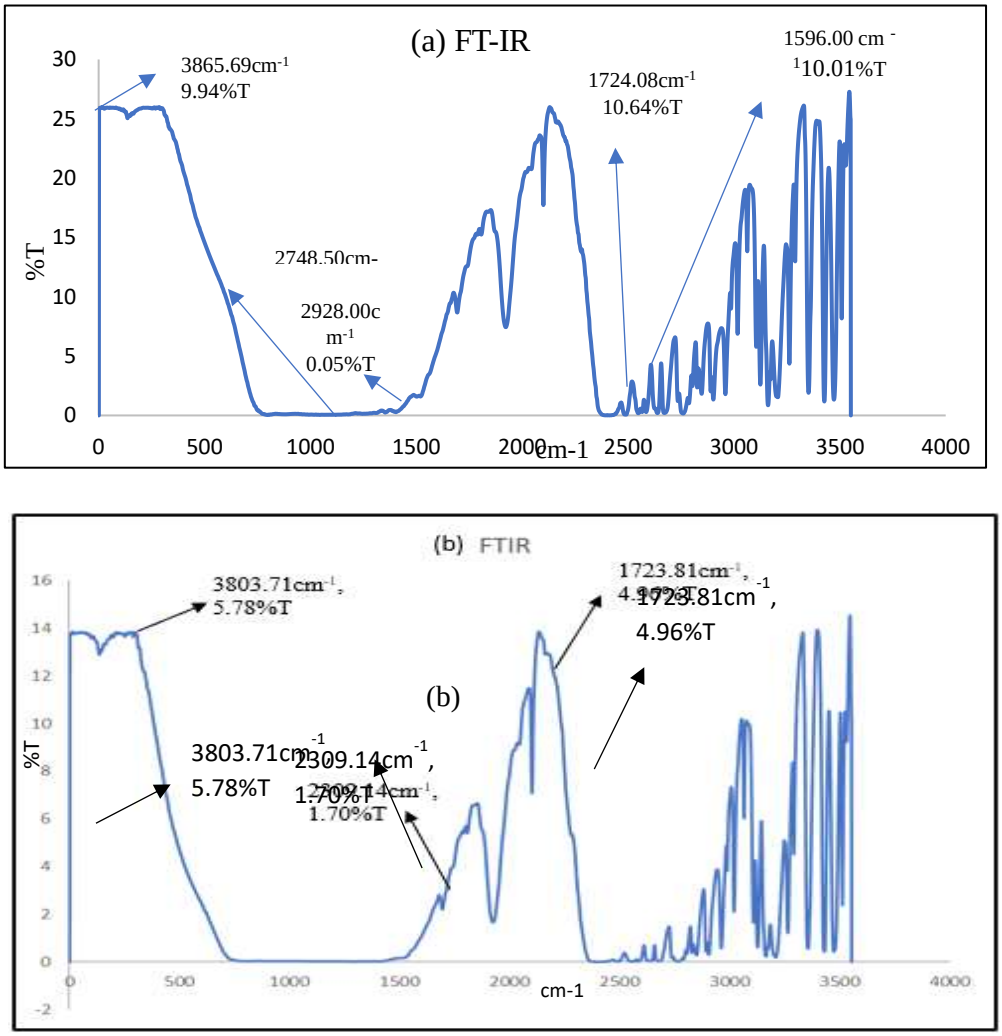


Figure 7. FT-IR spectrum of purified PHB produced by (A) *Bacillus subtilis* and (B) *Bacillus cereus* using Tyrosine as Amino acid Sources

The functional groups of the extracted PHB granules were identified as O-H, N-H, C-H, C=C, and C=O groups by FT-IR spectroscopy the results of FT-IR spectroscopy are shown in Figure 7. Moreover, the band at 3865.71 cm−1 indicates the stretching of the O–H group, whereas the peak at 2748.50 cm−1 is assigned to the C–H bond. Furthermore, the spectra revealed the appearance of the band at around 1724.08 cm−1, which is matched with the C=O group, the peak at 2928.00 cm−1 indicates the N-H bond. The absorption bands at 1596.00 cm−1 are associated with the presence of C–O group. The IR spectroscopic analysis gave further insights into the chemical structure of the polymer and reflects the monomeric units. In this study, the functional groups of the polymer PHB were confirmed as O-H, N-H, C-H, C=O, and C=C groups by FT-IR spectroscopy.

Table 3. Presence of Functional Group in the Production of PHB

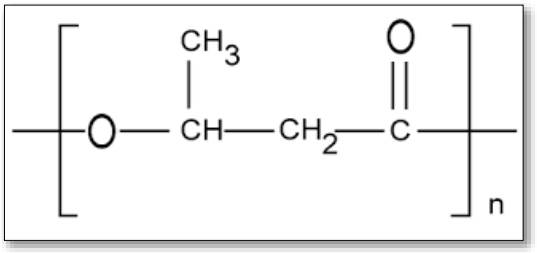
GROUPS PRESENT IN PHB	
OH	
$CH3$	
CH	
$CH2$	
$C = O$	Structure of Polyhydroxy butyrate
Functional Groups Present in PHB	

Table 3 represents the frequencies, appearance, functional group and compound class present in PHB during Fourier Transform infrared spectroscopy.

Table 4. Presence of Functional Group in the Produced PHB from *Bacillus spp.*,

S.NO	Group Frequencies	Appearance	Assignment of Function group	Compound class
1.	3865.71 $cm-1$	Strong, Broad	O-H Stretching	Alcohol
2.	2928.00 $cm-1$	Strong, Broad	N-H Stretching	Amine salt
3.	2748.50 $cm-1$	Medium	C-H Stretching	Alkane
4.	1724.08 $cm-1$	Strong	C=O Stretching	Anhydride
5.	1596.00 $cm-1$	Strong	C=C Stretching	Alkene

Table 4 signify the frequencies, appearance, functional group and compound class present in PHB Produced from *Bacillus subtilis* and *Bacillus cereus* during Fourier Transform infrared spectroscopy.

4.1 Nuclear Magnetic Resonances

NMR analysis was carried out for the sample of PHB which produced by 10g of Tyrosine. Bruker manufacturer of 300 MHz – FT- NMR Spectroscopy was used to analysis the sample. The figure 8 represents the peak values obtained from tyrosine as an amino acid source in proton nuclear magnetic resonance spectroscopy. The range obtained are in 7.344 ppm for C-H stretching, 1.635 ppm for C=O stretching and 1.4 ppm for C=C stretching. That confirms the production of PHB from Tyrosine as amino acid and also confirms the presence of components of PHB.

5. Conclusion

The availability of amino acid sources plays a crucial role in the efficient production of specific metabolic compounds. In-depth studies were conducted to investigate the impact of amino acid sources on the growth rate and production rate of Polyhydroxybutyrate (PHB). Through *in-silico* analysis of the core metabolic model of *Bacillus subtilis*, it was determined that tyrosine had the most significant effect on PHB production. The expected results using tyrosine as an amino acid source yielded an FBA objective function value (fval) of 0.3010. Subsequent in vitro analysis of growth rate and PHB production rate validated the findings, demonstrating that a high concentration of tyrosine is an effective amino acid source for PHB production. In *Bacillus subtilis*, it resulted in the production of 0.9 g/ml of residual biomass and 50.0 % PHB.

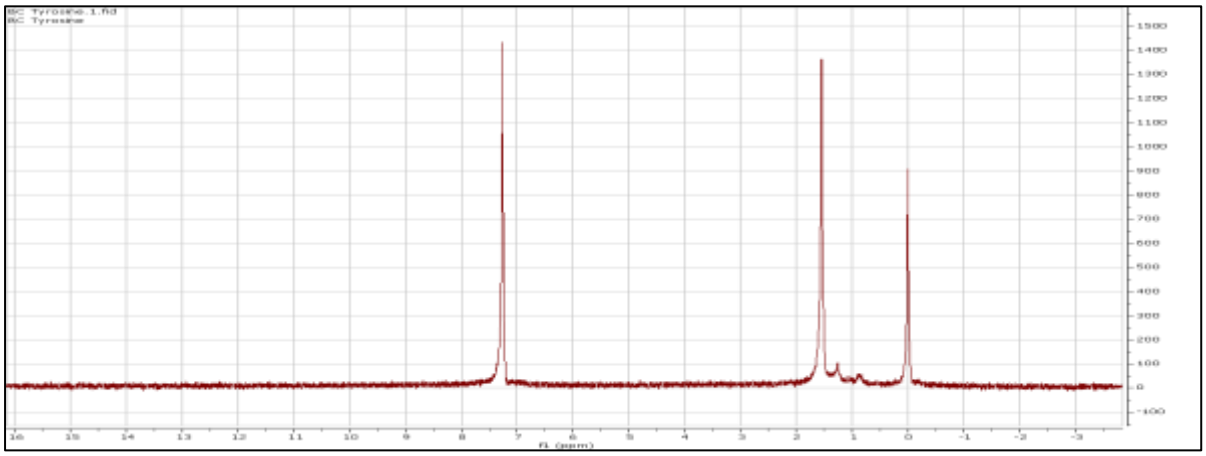


Figure 8. NMR peak for the Amino Acid source (Tyrosine)

Similarly, in *Bacillus cereus*, the use of tyrosine as an amino acid source led to even higher PHB production, with 0.46 g/ml of residual biomass and 61.34 % PHB. This work concludes that Flux balance analysis (FBA) can serve as a valuable tool for enhancing PHB production. The results indicate that leveraging FBA for strain design holds great potential to achieve breakthroughs in traditional metabolic engineering strategies, opening up new possibilities for optimizing PHB production and related process. There are many uses for biodegradable plastic, including the development of farming and building materials, car interior materials, electronic devices, bottles, and receptacles, among others. PHB has applications in medical and pharmacological fields like surgery sutures, wound dressings, and eye devices because it is compatible with human parts. PHB can be used as a food packaging material.

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