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FLUX BALANCE ANALYSIS FOR MAXIMIZING POLYHYDROXYALKANOATE PRODUCTION IN *PSEUDOMONAS PUTIDA*

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ABSTRACT

The work is aimed at developing process strategies to understand the behavior of *P. putida* for cost effective synthesis of mcl-PHA with the help of metabolic flux model developed using linear programming. With this context, screening and selection of suitable carbon sources for mcl-PHA production was done. Among the various carbon sources used for shake flask experiments, linoleic acid gave maximum product synthesis than glucose and glycerol. The higher yield in linoleic acid is not reported in literature so far. Metabolic flux analysis was done in nitrogen limited continuous cultivation of Pseudomonas putida with glucose, glycerol and linoleic acid as carbon sources. The reasons for higher PHA biosynthesis in linoleic acid can be understood by the metabolic flux analysis of continuous cultivation experiments with various carbon sources. The optimal C/N ratio was obtained by simulating with the range of carbon and nitrogen uptake rates as constraints of the metabolic flux model. The model predicted optimal C/N ratio and the same was implemented in fed batch cultivation experiments. The fed batch fermentation resulted the highest PHA synthesis with linoleum acid as carbon source. But the cost of linoleic acid is the tailback for the cost effective production of mcl-PHA. A metabolic flux model was developed for dual substrate utilization to minimize the amount of linoleic acid for optimal PHA synthesis. Atlast, the two stage fed batch fermentation strategy was implemented with glucose and linoleic acid for cost effective biosynthesis of PHA.

Key words: mcl-PHA, Metabolic flux analysis, Continuous cultivation, Two stage fed batch fermentation, Dual substrate, Simulation.

INTRODUCTION

Polyhydroxyalkanoates (PHA) are biopolymers stored by a wide variety of organisms as an energy reserve[1,2]. There were two major factors that eventually brought upon the PHA a widespread industrial interest. Firstly, the world oil crisis in the early 1970s resulted in the instability of oil market and oil shortages. Secondly, a growing interest in biodegradability and sustainability in the 1980s and early 1990s. PHA being biodegradable and biocompatible have an application varies from industries to medical therapeutics.The microorganism accumulate PHA as an intracellular reserved material in response to the imbalance in the growth environment,

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where a suitable carbon source is present in excess and one or more nutrients are limiting e.g. nitrogen, oxygen, phosphorus, iron, magnesium, manganese, potassium, and sodium or when the C : N ratio of the feed substrate is high, an excess of carbon source with deficiency in trace elements etc.[2-6] Metabolic flux analysis is the method to evaluate the amount of intracellular metabolites flow in the central carbon metabolism, from the knowledge of rate of production of extra cellular products, rate of substrate utilization, rate of product formation, rate of biomass production etc. In this work the detailed study of metabolites flow towards mcl-PHA biosynthesis was done with glucose, glycerol and linoleic acid as carbon sources. This work is aimed at developing process strategies for enhanced and cost effective synthesis of mcl-PHA with the help of insilico metabolic flux model developed using linear programming.

EXPERIMENTAL

Materials and methods

All chemicals, reagents and medium components used were of analytical grade. Standard reagents and medium components including glucose and inorganic salts were obtained either from Merck & Co (New Delhi, India) or Himedia Laboratories (Mumbai, India).

Strain and media used

Pseudomonas putida MTCC 102 (type B), a gram-negative bacterium was used throughout the study, obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India.

Two types of media were used to culture *P. putida* MTCC 102 (type B) viz.,

(i) A rich medium (Hori et al. 1994) primarily for promoting biomass growth.

(ii) A defined medium has been used as the basal medium for PHA production.

Analytical procedures

Estimation of various extra cellular metabolites produced during continuous fermentation by high performance liquid chromatography (HPLC)

The carbon sources and various extracellular metabolites were quantitatively estimated using HPLC equipped with Aminex HPX-87H column and Refractive Index detector. Prior to metabolites estimation in the fermenter broth, the standard graphs of metabolites like citric acid, succinic acid, lactic acid and acetic acid were prepared from different concentrations of each metabolite and their corresponding area under the peak. The amount of carbon sources and metabolites like acetic acid, lactic acid, citric acid, succinic acid and ethanol were analyzed quantitatively during fermentation.

Fourier transform infra-red spectroscopy (FTIR)

Fourier-Transform Infra-Red spectroscopy (FT-IR) has been demonstrated to be a powerful tool for studying microorganisms and their cell components in intact form[7-9]. In this

study the extended observation was done to detect mcl-PHA by the FT-IR technique in intact cells and also in purified form.

Purification and measurement of the PHA from biomass

The purification procedure was used for PHA extraction was as described earlier[10]. Total PHA content is the ratio of the total amount of polymer obtained after final purification by methanol precipitation to the weight of the dried cells. It was found that the percentage of recovery was not affected by the presence of residual biomass and the same has been mentioned[11].

Optimization using linear programming by GAMS

The flux analysis can be performed with the help of GAMS. The General Algebraic Modeling System (GAMS) is modeling system for mathematical programming and optimization of objective of interest, particularly designed for modeling linear, nonlinear and mixed integer optimization problems, wherein linear programming (LP) is a technique for optimization of a linear objective function, to achieve the best outcome in a given mathematical model (here GAMS) subject to linear equality and linear inequality constraints.

RESULTS AND DISCUSSION

Since glucose, glycerol and linoleic acid engage different pathways for synthesis of PHA, the study was carried out with these carbon sources. Further, in these carbon sources, Glucose is catabolized through ED pathway while glycerol is catabolized by glycerolipid metabolism and linoleic acid by β-Oxidation pathway for growth and biosynthesis of PHA.

From the batch cultivation with glucose, glycerol and linoleic acid it was found that the process needs to be developed, since C/N ratio is crucial, it also suggests a higher C/N ratio to be adopted or fed batch startegy in which continuous supply of carbon essential for PHA synthesis needs to be implemented.

Confirmation of mcl-PHA biosynthesis by FTIR analysis

FTIR analysis is the one of accurate method for the detection of PHA in intact cells or in purified PHA. The FT-IR method does not require extensive sample preparation and therefore it is also very useful for broad screening of PHA producing microorganisms. Hong developed a rapid method for detecting bacterial Polyhydroxyalkanoates both in intact cells and purified form by Fourier transform infrared spectroscopy[12].

The FTIR was done and the bands observed in the FTIR spectra (Fig. 1) is similar to the bands observed in the spectra reported by Hong et al.[12] Totally 4 bands corresponding to mcl-PHA was obtained they were at wave number's 1740.44, 2859.92, 1069.33 and 2924.52. The obtained FT-IR spectrum confirms the presence of mcl-PHA (Fig. 1).

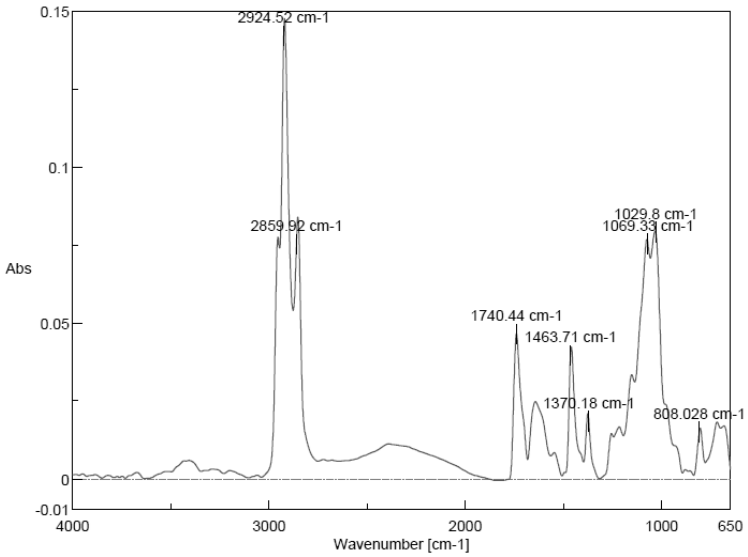

Fig. 1: Fourier-transform infrared (FT-IR) spectra of pure mcl-PHA extracted from *P. putida*

The four peaks of the FTIR spectra shown is similar to the peaks obtained by Hong et al.[12] The first three bands confirm the presence of mcl-PHA and the last band describes the methylene C-H vibration of mcl-PHA as it is also observed by Hong et al.[12]

Simulation of optimal c/n ratio for the higher yield of mcl-PHA using metabolic flux model

In the case of batch experiments, availability of excess carbon and limiting nitrogen till the end of log phase is crucial for the synthesis of PHA during the stationary phase. C/N ratio of 15.15 was chosen based on literature report.[13] However from the results of batch experiments, it was found that exhaustion of glucose during log phase, while nitrogen being available till the middle of the stationary phase. Therefore it is decided to choose a higher C/N ratio of 37.87 (w/w) for chemostat studies. Further, needed optimal C/N ratio for maximum PHA synthesis in fed-batch culture. Therefore to minimize the number of experiments, the metabolic flux model was simulated for various C/N ratios to find the optimal value for maximum PHA biosynthesis (Fig. 2). The carbon to nitrogen ratio was varied by altering the constraints, the fluxes for carbon source uptake rate and ammonia uptake rate. The model suggested an optimal value of around 75.75(w/w). Hence the same ratio was used in our fedbatch experiments. In Fig. 1 there was steep increase in the quantity of PHA synthesized around C/N ratio of 75.75.

Continuous cultivation studies of pseudomonas putida in glucose, glycerol and linoleic acid as carbon sources

Metabolic flux analysis was done at steady state in chemostat experiments to understand primarily the regulation involved in PHA synthesis and flux flowing through various pathways.

Further, it would lead to identification of rate limiting steps based on flux analysis which would lead to identification of targets for rational metabolic engineering to increase the PHA synthesis rate.

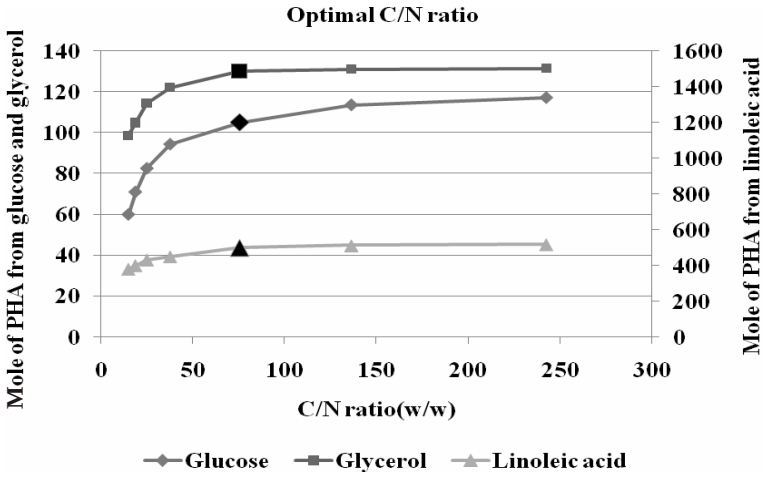

Fig. 2: Simulation of various C/N ratio for higher yield of PHA using metabolic flux model

The growth profile of the culture during transient state was followed by steady state . Nearly four reactor volume (5.6 L) of the medium was fed to achieve steady state. Steady state in the reactor was identified by the steady state cell concentration, residual substrates and various other secreted metabolites in the culture broth. The steady state was maintained for 20 h to have reliable output. The carbon source concentration of 10 g/L was chosen as it was not inhibitory to growth in the shake flask and batch experiments. The specific growth supported by linoleic acid is least and was found to be 0.1/h. In order to analyze the metabolic flux for these different carbon sources at the same dilution rate and to prevent the wash out, a dilution rate of 0.06/h was arbitrarily chosen for the experiments.

The rate of biomass growth supported by these three different carbon sources are different. Growth on glucose was much faster than on glycerol and linoleic acid. The latter supported the slowest growth rate. Later during the steady state the biomass concentration decreased gradually and attained a constant value which is dependent on the biomass yield with respect to various carbon sources. The steady state value of biomass concentration provides a measure of the biomass growth supported by the carbon sources. Glucose supported the maximum biomass and hence yield followed by glycerol and linoleic acid. These observations suggest that glucose is the preferred carbon source for increasing the cell density in fed batch experiments.

The analysis of continuous cultivation experiments revealed interesting insights. Analysis of the various extra cellular metabolites which are the byproducts at steady state are

shown in Table 1. The metabolites detected by HPLC for glucose as carbon source were citric acid, lactic acid, acetic acid, succinic acid and ethanol whereas on glycerol succinic acid and ethanol are the two metabolites secreted. For linoleic acid as carbon source, the presence of citric acid alone was found.

Continuous fermentation experiments with various limiting nutrients like N, P, Mg, and Fe, has been done and found that PHA production increasing significantly, when nitrogen was used as a limiting nutrient[14]. The nitrogen limitation resulted in redirection the carbon flux towards mcl-PHA biosynthesis. Therefore, in the experiments nitrogen is used as the limiting source in chemostat experiments.

Table 1: Rate of formation of extracellular metabolites and mcl-PHA for various carbon sources in nitrogen limiting continuous fermentation

Carbon source	Acetic acid (mmole/ g/h)	Citric acid (mmole/ g/h)	Ethanol (mmole/ g/h)	Lactic acid (mmole/ g/h)	Succinic acid (mmole/ g/h)	mcl-PHA (mmole/ g/h)
Glucose	5.2×10^{-3}	1.95×10^{-3}	0.0489	3.714×10^{-3}	6.3×10^{-4}	8.32×10^{-5}
Glycerol	-	-	0.047	-	4.44×10^{-5}	1.22×10^{-5}
Linoleic acid	-	1.51×10^{-3}	-	-	-	2.59×10^{-4}

Metabolic flux analysis for the continuous cultivation experiments for three different carbon sources

The central metabolic pathway for three carbon sources varies in many aspects like the pathway used for catabolism of carbon sources and production of extra-cellular metabolites. From the flux maps (Figs. 3-5) and the growth profile of the three different carbon sources in chemostat we observed the following things. The following observations were observed for the given constraints and growth conditions. The values vary with the conditions used for simulation. In *Pseudomonas putida* glucose is processed through glycolysis and subsequently through Entner Doudoroff pathway (Fig. 3). The flux for glucose branched into ED pathway and PP pathway, with 85 : 15 ratio. Of the 160 mole of intermediates generated from glucose, around 130 mole of Pyruvate is formed. 30 mole is channeled through anaplerotic pathway to replenish TCA cycle intermediate oxaloacetate.

Around 100 mole of pyruvate is converted into acetyl CoA and the remaining 30 mole is not accounted in our flux analysis. The acetyl CoA flux is distributed to respiratory activity and fatty acid biosynthesis pathway in the ratio of approximately 40:60. The cell allocates around $1/5^{th}$ to mcl-PHA formation and remaining is utilized for making lipids for cellular needs. Nearly

half of the flux flows in TCA cycle and utilized for the production of extracellular products like citric acid and succinic acid, urea cycle, amino acid biosynthesis pathway and biomass production. Negligible amount of ethanol acetate, lactate and citrate is formed which accounts for less than two 2% of the flux.

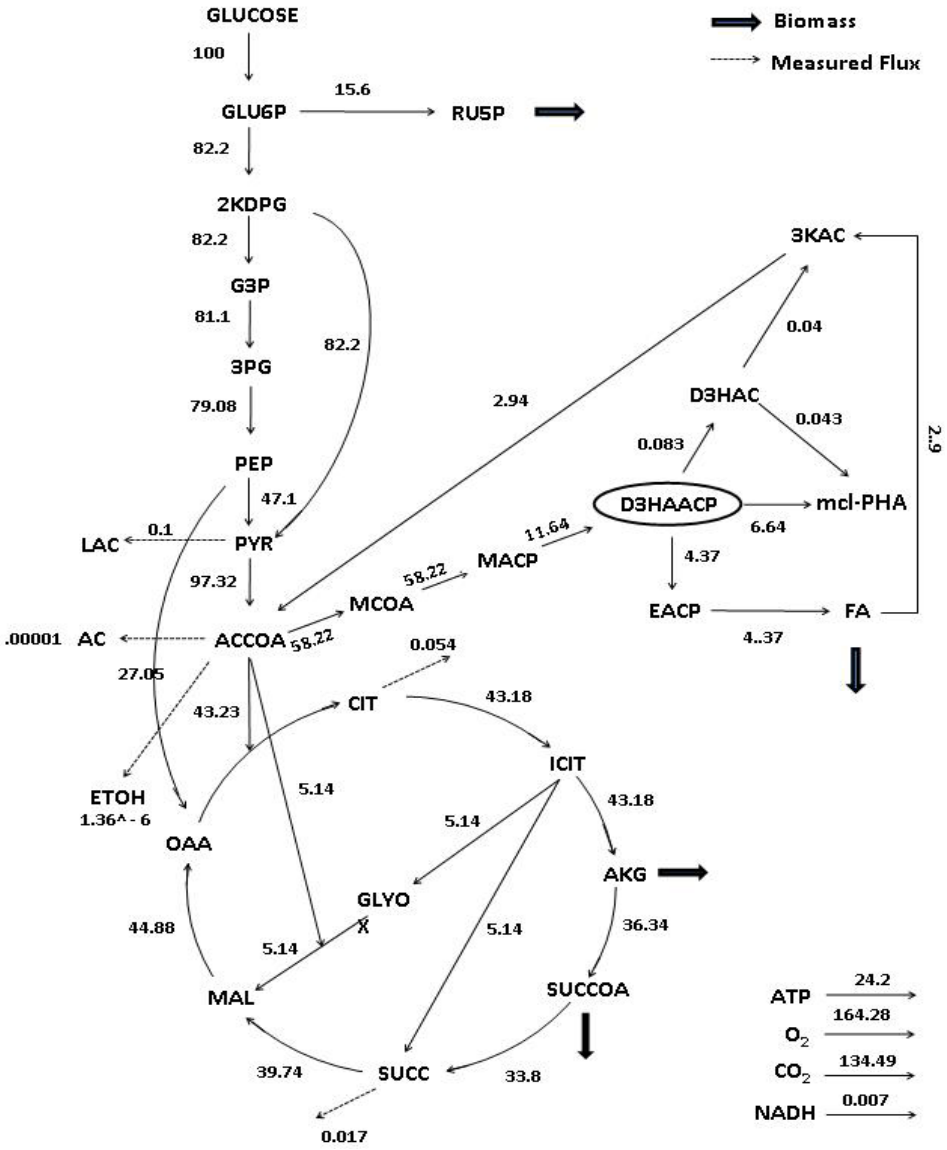

Fig. 3: Metabolic flux map of glucose used as a sole carbon source

Glycerol as a solitary carbon source is metabolized through different pathway when compared to glucose catabolism (Fig. 4). Glycerol undergoes glycerolipid metabolism to convert

glycerol to pyruvate. Of the 100 mole of glycerol utilize roughly 70% of it is utilized for gluconeogenesis and PP pathway. The remaining 30% of flux is metabolized to make Pyruvate with a fraction of it being sequestered for anaplerosis. Majority of the acetyl CoA formed from pyruvate is distributed to fatty acid biosynthesis with negligible respiratory activity.

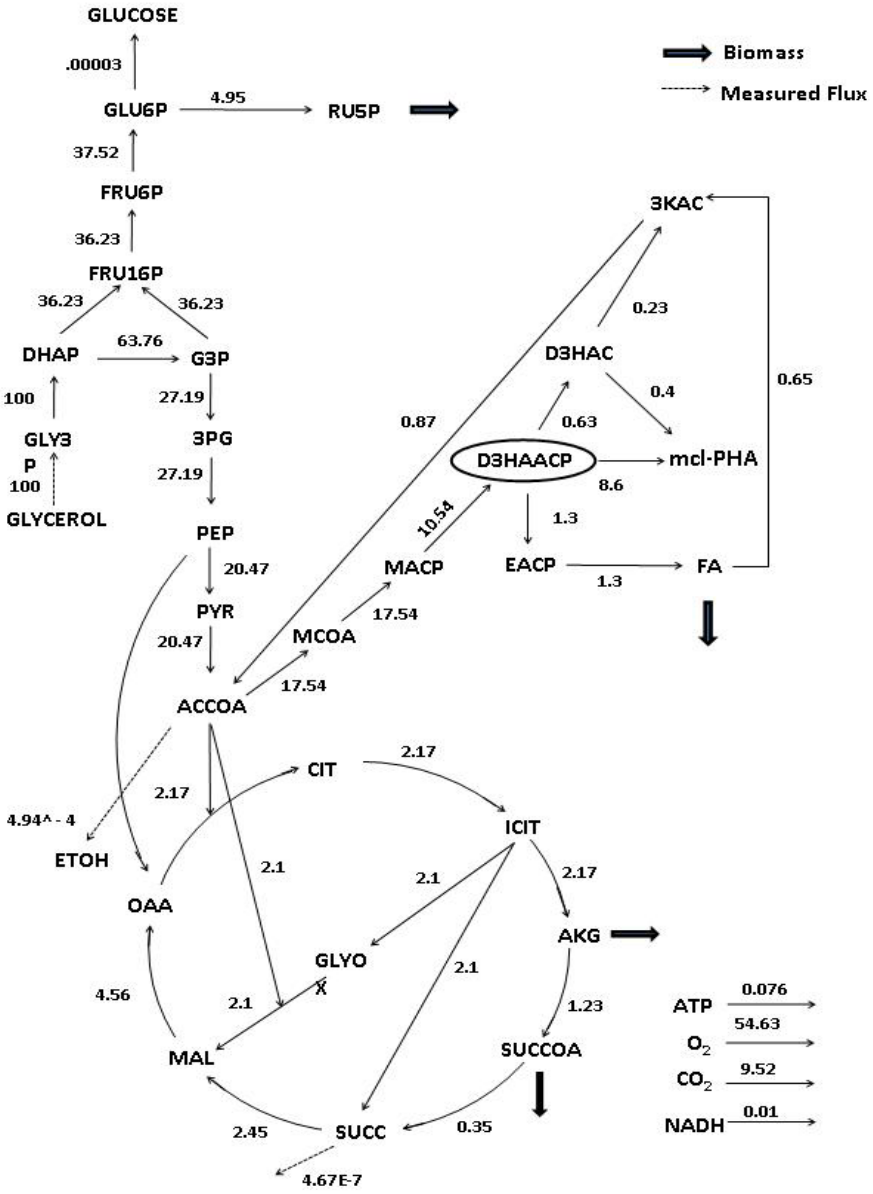

Fig. 4: Metabolic flux map of glycerol used as a sole carbon source

β-Oxidation pathway is the predominant pathway for the catabolism of linoleic acid as a sole carbon source (Fig. 5). The linoleic acid can be utilized through β-oxidation pathway and enters into TCA cycle by means of 3-ketoacylCoA by forming acetyl CoA as an intermediate. Of the 100 mole of linoleic acid consumed, 75 mole utilized in β-oxidation pathway and the remaining 25 mole of flux utilized for the biosynthesis of mcl-PHA. Of the 75% of flux is directed towards acetyl-CoA and from acetyl-CoA nearly 55% of flux utilized in respiratory cycle. The remaining flux is used for making pyruvate from oxaloacetate, extracellular citric acid, gluconeogenesis and PP pathway.

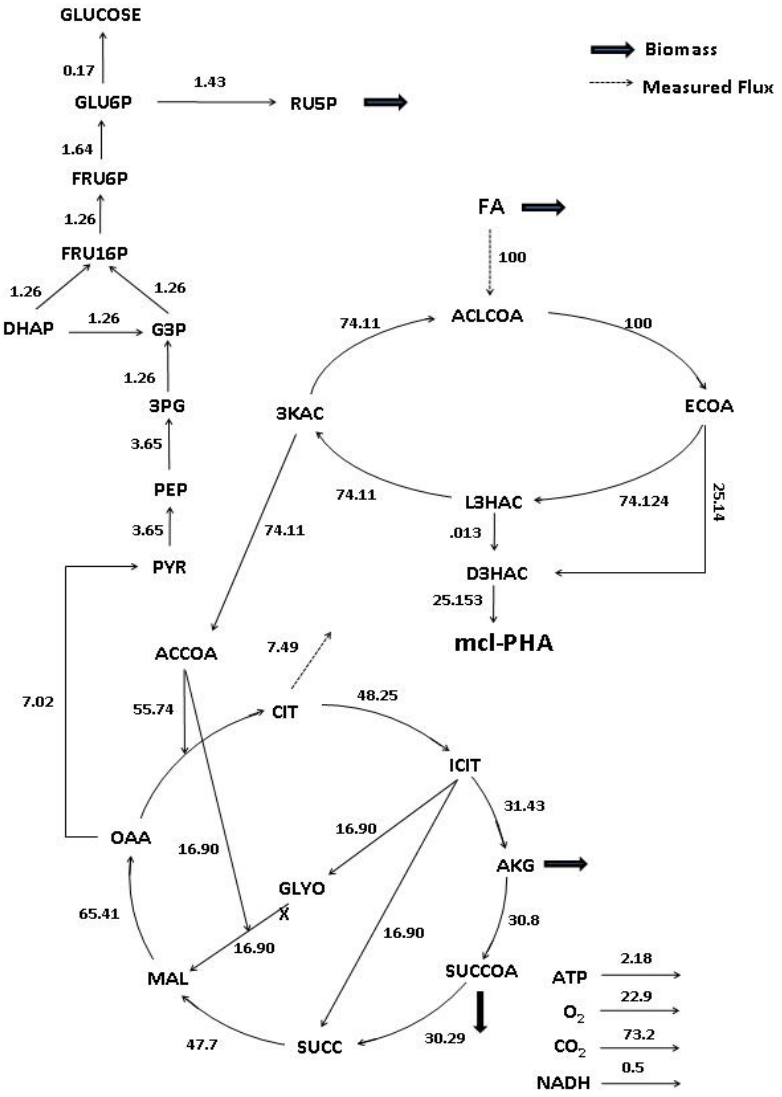

Fig. 5: Metabolic flux map of linoleic acid used as a sole carbon source

To enhance the synthesis of PHA for glucose feed, the fluxes of acetyl-CoA to the citric acid cycle have to be minimized. This may be achived by altering the enzyme concentration of citrate synthase or minimizing the anaplerosis by targeting pyruvate carboxylase.

It is well known that ATP is responsible for cell maintenance and as an energy resource for most of the pathways. The ATP produced must be dissipated by either biomass production or the hydrolysis of ATP in pathways not related to PHA synthesis. The ATP generation was high in the case of glucose feed when compared to glycerol and linoleic acid. The higher ATP generation is due to active respiration resulting in CO_2 generation. As a result, the biomass yield should decrease. This can be seen in batch and chemostat experiments, while glycerol and linoleic acid led to synthesis of more biomass compared to glucose. Higher specific growth rate in glucose feed may have been due to higher ATP generation when compared to other two carbon feed. Therefore, inactivation of the glyoxylate pathway and down regulation of isocitrate dehydrogenase should result in an increase in the overall flux of acetyl-CoA into mcl PHA production.[15]

Fed-batch fermentation with glucose, glycerol and linoleic acid as carbon sources

Quasi steady state exponential fed batch was done with the three carbon sources, glucose, glycerol and linoleic acid. Exponential feeding was implemented to achieve high cell density and higher PHA biosynthesis in all fed-batch fermentations with feed concentrations of 50 g/L of carbon substrate and 0.66 g/L of ammonium sulphate as a nitrogen source. The optimal C/N ratio, 75.75 (w/w) indicated by the simulation results is implemented. The desired specific growth rate of 0.1/h is incorporated into the feed forward model and was used in all fed batch fermentations.

The feeding was started based on the observations made in batch cultivation. Experimental feeding was continued until biomass reached an OD_{600} of 60 for glucose, 80 for glycerol and 100 for linoleic acid. Biomass increased further and reached stationary phase. After reaching the stationary phase the reactor was allowed to continue for 2-4 h as PHA will be accumulating in the stationary phase.

Linoleic acid produced more amount of mcl-PHA as compared with glucose and glycerol. Yield of 61% (w/w) is one of the highest reported in the literature for mcl-PHA production. As predicted from the model, C/N ratio of 75.75 may be the reason for increased amount of PHA synthesized in fed batch cultivation compared to batch and continuous experiments.

Simulation of dual substrate metabolic flux model

A metabolic flux model with simultaneous utilization of glucose and linoleic acid was developed. The ratio of glucose and linoleic acid was varied as shown in the Fig. 6.

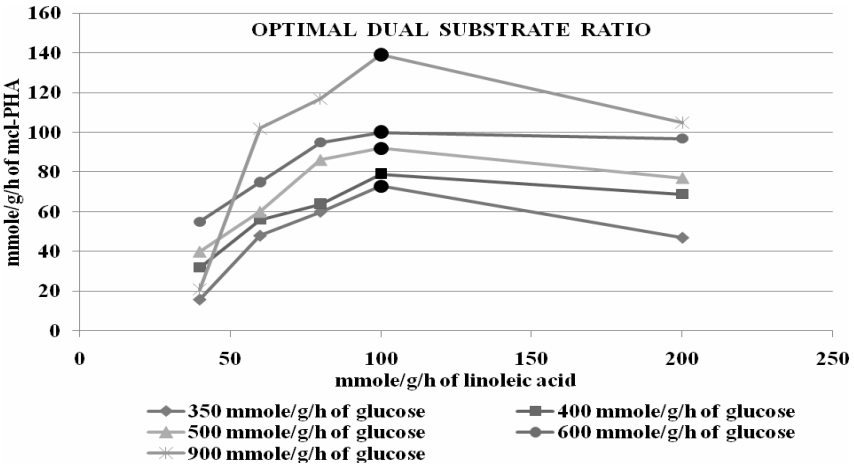

Fig. 6: Simulation of dual substrate metabolic flux model

For co-feeding ratio 1:1 of glucose and linoleic acid, the model (Fig. 7) predicted that most of the linoleic acid utilized for making PHA while 10% of PHA is made from glucose. Hence this insilico analysis gives an insight for the cost effective production of mcl-PHA. The model predicted that most of the mcl-PHA synthesized only from β-oxidation pathway and very less fraction of flux was obtained from glucose.

Further ratio of glucose to linoleic acid cofeeding was varied in the dual substrate metabolic flux model. This was done by varying the constraints for glucose uptake rate and linoleic acid uptake rate. The PHA synthesize rate changed for 40 to 200 mmole/g/h of linoleic acid and for various uptake rates of glucose ranging from 100 to 900 mmole/g/h. PHA synthesis rate was maximum in all the case for 100 mmole/g/h of linoleic acid feed and is found to be increasing with glucose uptake rate till 900 mmole/g/h. However experimentally the glucose uptake rate of around 350 mmole/g/h was observed. Therefore, a maximum PHA monomer production of 73 mmole/g/h was predicted for 100 mmole/g/h of linoleic acid.

Two stage fermentation system with glucose and linoleic acid

In order to reduce the cost of the substrate, two stage fed batch cultivation was envisaged. Cheap carbon sources can be used as feed for increasing the cell concentrations and subsequently linoleic acid as a feed for enhancing PHA biosynthesis. A combination of glucose and linoleic acid feeding during PHA synthesis phase in fed batch cultivation would result in more linoleic acid being used for PHA synthesis, while the cellular needs for biomass synthesis and energy generation will be borne by glucose.

The amount of glucose and linoleic acid used in two stage fed batch fermentation experiments were in the ratio of 1 : 5. At the time of starting of 2.5 g/L linoleic acid feeding, the residual glucose was nearly 2 g/L and the pulse of 10 g of linoleic acid was fed every hour. The reactor was started as batch with the initial glucose of 10 g/L, after 10 h of cultivation, residual

glucose concentration found to be negligible (Fig. 8) and the OD_{600} measured was 24.5. Exponential feeding with glucose (50 g/L) was continued upto the cell concentration reached OD_{600} of 65.6 started at the early stationary phase (PHA accumulation phase), the OD_{600} of 65.6 was reached corresponding to 10.45 g/L of biomass achieved and the glucose feeding was terminated. Subsequently linoleic acid was pulse fed.

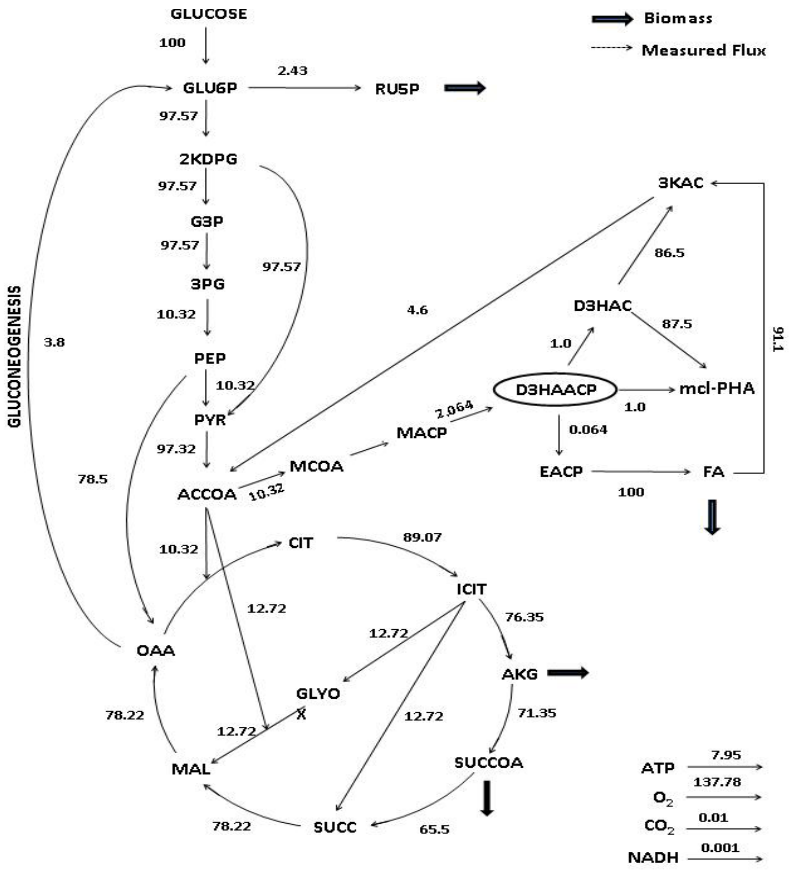

Fig. 7: Simulated metabolic flux map of glucose and linoleic acid cofeeding with 1:1 molar ratio

During the second stage of fed batch cultivation, the residual glucose of 2g/L was gradually co-utilized along with linoleic acid which was pulse fed at the rate of 2.5 g/h for 4 h. Co-utilization of glucose and linoleic acid have resulted in a final biomass concentration of 18.5 g/L and PHA yield of 51% (w/w). Since residual linoleic acid concentration not measured the linoleic acid uptake rate could not be measured and therefore, the optimal ratio predicted from the model could not be implemented. However continuous cofeeding of glucose and linoleic acid with various ratios could result in optimal PHA synthesis. Further work needs to be done in this direction.

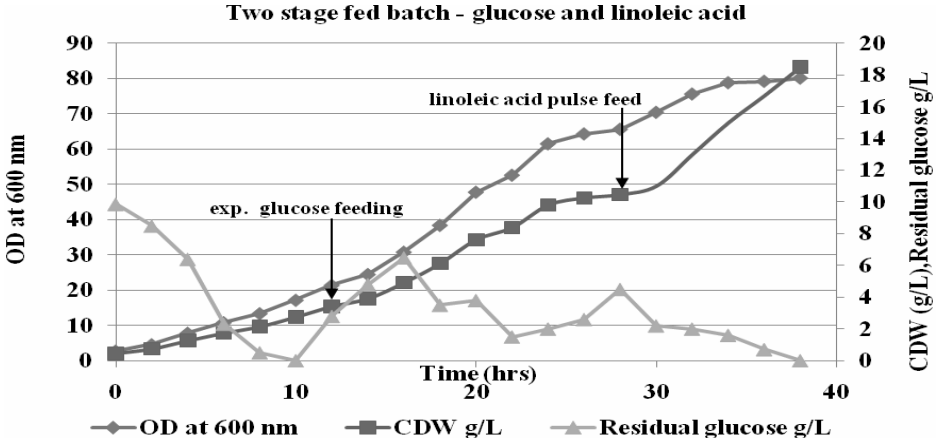

Fig. 8: Profile of two stage fed batch fermentation with glucose and linoleic acid

Comparison of product yield in single stage and two stage fed batch fermentation

The comparison of PHA yield in single stage and two stage fed batch fermentation with linoleic acid is playing a vital role towards cost effective biosynthesis of PHA. The Table 2 compares the total amount of linoleic acid fed, total PHA obtained and PHA product yield coefficient with respect to substrate in both single and two stages fed batch fermentation.

Of the total 60 gram of linoleic acid, 16.83 gram of PHA is obtained in linoleic acid in fed batch fermentation but in two stage fermentation 9.435 gram of PHA is obtained from 10 gram of linoleic acid. Here the contribution of glucose in PHA synthesis is not mentioned because the cost of glucose is less when compared with linoleic acid. The yield coefficient of two stage fermentation increased more than three folds than in single stage fed batch. Two stage fed batch fermentation with glucose and linoleic acid is better than the single stage fed batch fermentation system for cost effective and enhanced PHA production.

Table 2: Comparison of product yield in single stage and two stage fed batch fermentation

Type of fermentation	Mass of linoleic acid fed (g)	Mass of PHA obtained (g)	Yield ($Y_{P/S}$)
Single stage fed batch fermentation	60	16.183	0.2679
Two stage fed batch fermentation	10	9.435	0.9435

CONCLUSION

Among the various carbon sources used for shake flask experiments, linoleic acid gave maximum product synthesis and is not reported in literature so far. When compared to glycerol and linoleic acid, glucose supported maximum specific growth rate in batch cultivation experiments in bioreactor. However, the maximum biomass achieved was higher in glycerol and linoleic acid. The reasons for higher PHA biosynthesis in linoleic acid can been explained by the metabolic flux analysis of continuous cultivation experiments with various carbon sources.

Metabolic flux model using linear programming was developed to predict the behaviour of *P. putida* for cost effective and optimal biosynthesis of PHA. This model development resulted in minimizing the number of experiments needed to achieve the objective. From the batch experiments and from literature, it was found that C/N ratio is crucial for higher PHA biosynthesis. Simulation was done with the model predicted optimal C/N ratio of 75.75(w/w) and has been implemented in the fed batch cultivation experiments. The yield of PHA was found to be more in fed batch when compared with batch and continuous cultivation experiments.

Multiple rounds of metabolic engineering may be essential to achieve product levels in glucose or glycerol comparable to the one achieved with linoleic acid. Therefore, a combination of glucose with linoleic acid as carbon substrates may be the cost effective strategy for PHA production. Hence, the two stage fedbatch fermentation strategy was implemented with glucose and linoleic acid for cost effective biosynthesis of PHA. A metabolic flux model for dual substrate utilization was developed to minimize the amount of linoleic acid utilized for optimal PHA biosynthesis. However more experiments needed to be done to verify the model and further enhance the productivity.

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