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OPTIMIZATION OF BACTERIAL POLYHYDROXYALKANOATE PRODUCTION USING ONE-FACTOR-AT-A-TIME APPROACH

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ABSTRACT

Polyhydroxyalkanoates(PHA), an ecofriendly alternative to synthetic plastics are synthesized by bacteria under unfavorable growth conditions. Its production can be controlled to a large extent by optimizing various influencing factors like type of microorganism, nutrient availability, environmental conditions of growth, *etc*. The present study was aimed to maximize PHA production by selected bacteria using one-factor-at-a-time approach(OFAT) using shake flask fermentation process. Six previously isolated bacterial cultures were identified by 16 S rRNA sequencing. Two bacteria identified as *Brucella melitensis* and *Microbacterium aurum* were found to be novel PHA producers. All isolates were optimized for physical and nutritional parameters which led to significant increase of 25-40% in the PHA yield as measured by gravimetric analysis. Three isolates namely *Bacillus flexus*, *Brucella melitensis* and *Pseudomonas aeruginosa* giving maximum PHA yield of 43.04 ±1.09, 47.38 ±1.31 and 49.06 ±0.75 % respectively after optimization and were found to be potential candidates for further optimization.

KEYWORDS: **Optimization, PHA, OFAT, shake flask, 16S rRNA, carbon to nitrogen ratio, *Brucella melitensis, Microbacterium aurum***

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INTRODUCTION

Petrochemical based plastics have caused menacing problems because of their threatening environmental effects along with the fast depletion of fossil fuels. [1,2]As a result, the research focus has shifted from synthetic plastics to produce and develop alternative biodegradable products. [3]Polyhydroxyalkanoates (PHA) have drawn considerable attention as suitable biodegradable plastics. [4,5] PHA are water insoluble polyesters and are considered as suitable substitutes for non-biodegradable polymers, not only due to their inherent biodegradability but also due to their other desirable qualities such as biocompatibility, incorporation of renewable resources as substrates in their production, material properties similar to polypropylene, *etc.*[6,7,8] These bio-polymers are stored intracellularly in the form of inclusion bodies and may get accumulated up to 80-90% of dry cell weight as reserve source of energy by some microorganisms.[4] These compounds are produced when growth medium composition is unbalanced due to factors like limitation of oxygen, nitrogen, phosphorous or sulfur and an excess of carbon source or when the C: N ratio of the feed substrate is higher.[9,10,11] Various microbial genera such as *Aeromonas, Alcaligenes, Azotobacter, Bacillus, Cupriavidus, Ralstonia, Rhizobium, Pseudomonas, Vibrio etc*. are known to have ability to produce PHA.[12,13] Accumulation of biomass along with PHA by bacteria is largely dependent on the species of bacteria and physical factors like temperature, pH, availability of oxygen, *etc*., along with nutritional factors like substrate availability and media composition.[14,15,16] In bacteria, PHA is synthesized from Acetyl-CoA by a sequential enzyme catalyzed process of 3 reactions starting with the enzyme 3-ketothiolase followed by aceto-acetyl CoA-reductase and lastly PHA synthase.[3] The composition and length of the PHA polymer is determined by the carbon source concentration of the growth medium and also the length of the carbon substrate.[3] Variation in carbon assimilation pathways and reducing power requirements among different bacterial species may influence polymer production. [17] PHA metabolism is controlled by the redox state of the cell and by the concentration of CoA-SH, pyruvate and 2-oxoglutarate and hence the type and concentration of carbon substrate and C:N ratio have maximum influence on the PHA accumulation.[18] The present work deals with the optimization of various physico-chemical parameters for maximum PHA production by some of the most promising bacterial cultures that were isolated from various habitats as reported earlier. [2] The parameters considered in the one-factor-at-a-time approach,carried out in the shake flask optimization study, were incubation temperature, pH of initial growth medium, agitation rate,time of incubation and nutrient medium constituents like type and amount of carbon source, type of nitrogen source and carbon-to-nitrogen ratio of the medium.

MATERIALS AND METHODS

Analytical grade chemicals were procured from Merck Pvt. Ltd., India and Sigma Aldrich Pvt. Ltd., India whereas bacteriological media were obtained from Hi-Media Pvt. Ltd., India.

Isolation and identification of the isolates

Bacterial cultures selected for the optimization study were isolated from varied sources and screened for their ability to produce PHA using primary method of Nile Blue A plate assay and secondary screening by UV-VIS spectrophotometry and flow cytometry as described by Wagle et al. (2015).[2] Isolation of the cultures coded as HP1, PNRN1 and UMB2 has been discussed previously by us.[2] The other 3 cultures coded as DYC3, GARS2 and EFF2 were isolated from dumping yard (Maharashtra Nature Park, Mahim Mumbai), garage soil(GTB Nagar, Sion Mumbai) and industrial effluent (Metallurgical Labs, Sewri, Mumbai), respectively. These 6 bacterial isolates were the highest PHA producers when quantified by the above mentioned techniques and hence were selected for optimization.The identification of all the 6 cultures was carried out using 16S rRNA sequencing using a multistep approach starting with bacterial genomic DNA isolation. Specific amplification of the 16S rRNA region of this template DNA was performed by PCR technique using 16S rRNA specific universal primers 27F and 1492R in a MJ Research PTC- 225 Peltier Thermal Cycler. The amplified segment was used as a template for performing sequencing using the 16S rRNA specific universal primers 785F and 907R. Sequencing was achieved using an Applied Biosystems ABI 3730xl sequencer. Sequence data was aligned and analyzed for identifying the isolates using the BLASTn search tool. The phylogeny analysis of sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. [19,20]

Seed preparation and growth conditions

Six cultures were grown in Luria Bertani broth on orbital shaker with temperature and agitation control (Scigenics Orbitek shaker model 400; stroke 25mm) at 150 rpm and 37°C overnight. Actively growing cultures (O.D 530nm adjusted to ~10^8 cells/cm^3) were inoculated (10% v/v) in 50 ml of M9 broth as PHA production medium in 250 ml Erlenmeyer flasks.[21] The growth conditions were changed one at a time as per the parameter to be optimized. All the experiments were carried out in triplicates.

Extraction of PHA

Ten ml of broth sample was centrifuged at 11,500 g for 10 min. The obtained pellet from the biomass was extracted for estimating PHA as per the modified method of John and Ralph, (1961). [22] Ten ml of acetone and ethanol (1:1 v/v) was used to wash the pellet prior to centrifuging and it was resuspended in 10ml of 4% sodium hypochlorite and incubated at room temperature for 45 min. The content was centrifuged and pellet was rewashed with acetone ethanol mixture (1:1 v/v). Finally the polymer fraction of the pellet was dissolved in 10 ml of boiling chloroform, filtered throughWhatman No.1.and the filtrate was evaporated to dryness to yield crude PHA powder.

Quantitation of PHA

The broth cultures were centrifuged at 11,500g to obtain the biomass pellet and dried to estimate the dry cell weight (DCW) and represented as g/L. [6,23] PHA yield was estimated as the difference between dry cell weight and dry weight of extracted PHA using the formula

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L)}}{\text{DCW (g/L)}} \times 100$$

Optimization of Culture conditions and media composition

Different factors influencing PHA production by the 6 bacterial isolates were optimized by varying all the conditions within a defined range using the One-Factor-At-A-Time (OFAT) approach. The shake flask fermentation was carried out as followed in secondary screening unless otherwise stated : incubation temperature of 37°C, medium pH of 7, agitation rate of 150 rpm and time of incubation 48 h.

Incubation temperature

The cultures inoculated in production medium were incubated under fixed agitation shaken condition at 4 different selected temperatures viz.20°C, 28°C, 37°C and 45°C for 48h taking into consideration mesophilic isolates.

Medium pH

For pH optimization, cultures were inoculated in production media adjusted to 4 different initial pH values i: e 5.5,6,7,8 based on previous evaluation.

Agitation rate

The production medium was inoculated with the selected isolates and incubated at 4 different agitation rates i:e 120 rpm,150 rpm,180 rpm and 210 rpm.

Incubation time

The inoculated medium was incubated for 5 different durations of incubation viz.24h,48h,72h,96h,120h.

Type of Nitrogen source

The isolates were grown in production media (1% C-source) incorporated with different nitrogen sources like Yeast extract, peptone, tryptone, ammonium chloride, ammonium sulphate and ammonium acetate at 0.3% concentration.

Type of Carbon source

The effect of different carbon sources on PHA production was studied by cultivating the isolates in production media supplemented with different carbon sources viz. glucose, sucrose, lactose, glycerol, mannitol, starch, maltodextrin, fructose-oligosaccharide(FOS) and tributyrin at 1% concentration along with the best nitrogen source at 0.3% concentration.

Concentration of Carbon source

The bacterial isolates were grown in production medium having the optimized nitrogen source (0.3%) concentration and best respective carbon source in different concentrations i.e. 0.5, 1.0, 1.5 and 2.0 %.

C:N ratio

The bacterial cultures were grown in nutrient medium with different C:N ratios i.e. 10:1, 20:1, 30:1 and 40:1 using the best C and N sources at optimized concentration of carbon source from previous study.

RESULTS AND DISCUSSION

Table I shows the 16S rRNA sequencing and BLAST search results post multiple sequence alignment. It can be observed that a close identity (99-100%) was obtained among the isolates under study and the already reported bacterial species. Notably isolates PNRN1 and GARS2 identified as *Brucella melitensis* strain AUH2 and *Microbacterium aurum* strain TPL18 respectively have not been reported earlier for PHA production.

Table I
Identification of isolates by 16S rRNA sequencing and BLAST search

Sr. No	Isolate Code	16S rRNA Sequence (bp Length)	Reference Sequence NCBI Accession No.	Percent Identity	Highest Similarity Hit By NCBI Blast Search
1	DYC3	1517	JQ936679.1	99	*Bacillus flexus* strain WY2
2	EFF2	1516	KF668463.1	99	*Bacillus subtilis subsp. inaquosorum strain IHB B 6833*
3	HP1	1498	KJ528948.1	100	*Pseudomonas aeruginosa* strain VSS6
4	PNRN1	1516	KF668463.1	99	*Brucella melitensis* strain AUH2
5	GARS2	1495	EU373396.1	99	*Microbacterium aurum* strain TPL18
6	UMB2	1527	JF513146.1	99	*Pseudomonas aeruginosa* strain S164S

The shake flask studies were initiated by first optimizing the physical factors affecting PHA production followed by the optimization of media components. From Fig. I it can be observed that a temperature of 37°C supported maximum PHA production for isolates *Bacillus flexus* WY2 (31.41±0.50%), *Pseudomonas aeruginosa* VSS6 (27.16±1.09 %) and *Pseudomonas aeruginosa* S164S (38.95±0.06%) whereas *Bacillus subtilis* 6833 (35.97±1.64 %), *Brucella melitensis* AUH2 (38.37±0.06 %) and *Microbacterium aurum* TPL18(25.6±0.95 %) yielded maximum PHA at lower temperature i.e. 28°C .These results are in agreement with those obtained by Aly et al.(2013), Elsayed et al.(2013) and Irsath et al.(2015). [24,25,26]

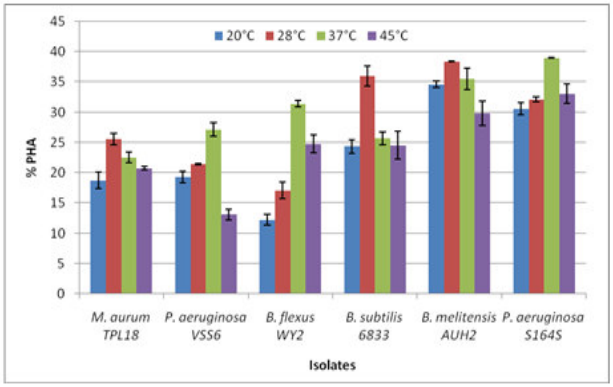


Figure I
Effect of temperature on PHA production

The influence of initial medium pH on PHA production as depicted in Fig.II indicates that isolates *Bacillus flexus* WY2, *Bacillus subtilis* 6833 , *Brucella melitensis* AUH2 and *Pseudomonas aeruginosa* S164S produced maximum PHA of 30.16±0.11, 27.32±0.17, 32.36±0.66, 35.14±0.12 % respectively at pH 7.0 while the isolates *Pseudomonas aeruginosa* VSS6 (25.02±0.47%) and *Microbacterium aurum* TPL18 (23.91±0.03%) produced highest amount of polymer at pH 6.0. These results are in accordance with the inferences drawn by Grothe et al. (1999), Panigrahi and Badveli (2013) and Aly et al. (2013) that a pH between 6.0-7.0 supports better polymer yield. [24,27,28]

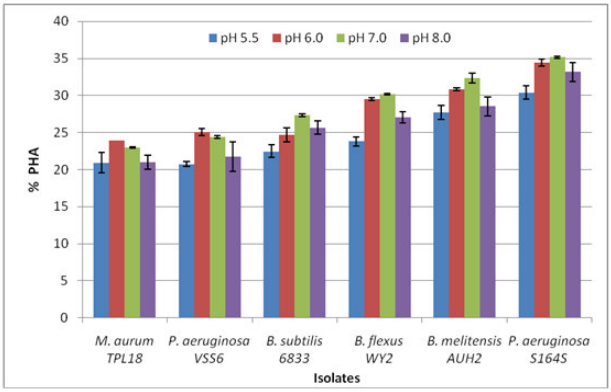


Figure II
Effect of pH on PHA production

Another factor that affects PHA production is availability of dissolved oxygen and hence the impact of agitation rate on the same was examined. [16] The results revealed that all isolates synthesized more PHA at shaker conditions of 150 rpm except *Microbacterium aurum* TPL18 for which optimum agitation rate was 180 (Fig. III). Upon further increasing agitation rate PHA production declined. From these results it may be inferred that stress created due to oxygen limitation at lower rpm led to increase in PHA production. Similar results have been reported by Wei et al. (2011) and Sav et al. (2014). [12,29]

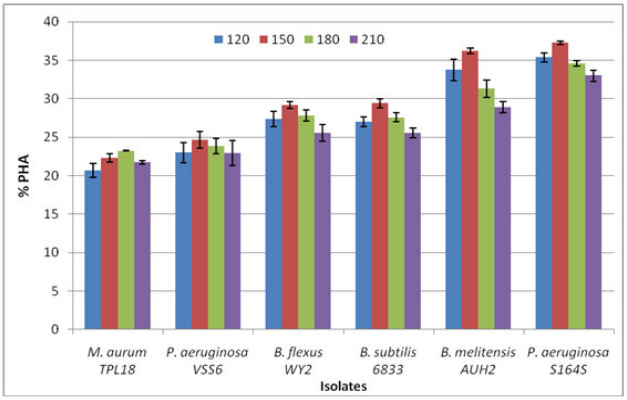


Figure III
Effect of agitation rate on PHA production

During optimization of incubation time, the bacterial cultures were grown at different time ranges from 24 hours upto 120 hours. From the data it was observed that PHA production increased with increase in incubation time yielding maximum PHA at 96 h for isolates *Bacillus flexus* WY2 (36.26±0.32 %), *Bacillus subtilis* 6833*(*38.43±1.01%*), Pseudomonas aeruginosa* VSS6 (32.09±0.09%) and *Microbacterium aurum* TPL18 (31.53±0.02%))while it was 72 h for *Brucella melitensis* AUH2 (40.85±0.23%) and *Pseudomonas aeruginosa* S164S (41.15± 0.65%) (Fig.IV). Post this incubation time the trend showed a decline in PHA yield which could be due to nutrient deficiency and/or rise in the levels of metabolites that might inhibit PHA production.[30] Earlier reports also show similar incubation durations for increased PHA production. [31,32,33]

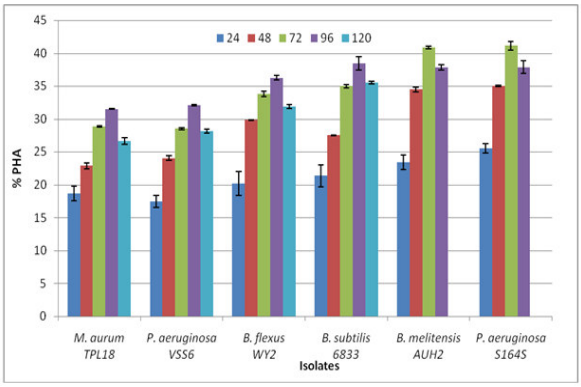


Figure IV
Effect of time of incubation(h) on PHA production

Organic nitrogen sources have been found to be superior to inorganic ones in terms of PHA production. [34] Similar findings were obtained in our study and are depicted in Fig. V. This result is in agreement with Rawte and Mawinkurve (2004) and Aly et al. (2013) who have also claimed yeast extract as the best nitrogen source for PHA production. [24,35] Use of peptone also resulted in higher polymer production which is in agreement with work reported by Shivakumar (2011) and Singh et al. (2011). [32,36]

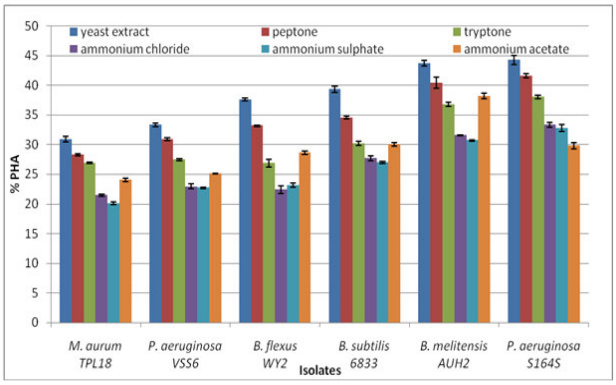


Figure V
Effect of nitrogen source on PHA production

Among the varied carbon sources tested to evaluate their effect on PHA yield, maltodextrin was found to be the best carbon source for the isolates *Bacillus flexus* WY2 (36.86±1.18%), *Bacillus subtilis* 6833*(36.07*±0.33%*), Brucella melitensis* AUH2 (41.03±0.62%), *Microbacterium aurum* TPL18 (32.06±1.07%) and tributyrin for *Pseudomonas aeruginosa* VSS6 (33.71±0.69%)and *Pseudomonas aeruginosa* S164S(40.63±1.11%) (Fig VI). Also FOS which gave PHA yield comparable to maltodextrin proved to be a promising carbon source across all the isolates studied. Earlier studies have reported use of simple sugars or other carbohydrates as carbon source for enhancing PHA production by bacteria. Commonly used prebiotics such as maltodextrin, FOS and a food grade flavor ingredient tributyrin have not been reported as carbon sources for improved PHA production.

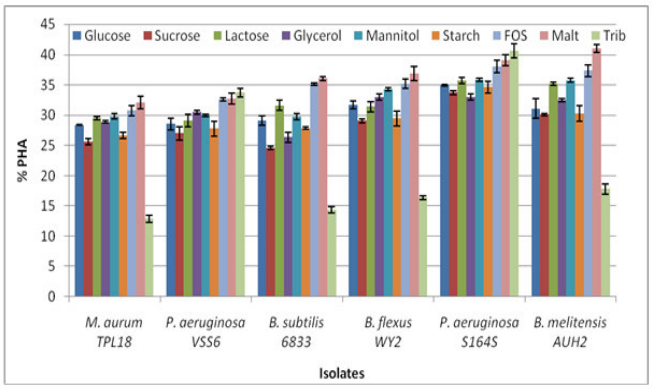


Figure VI
Effect of carbon source on PHA production

Carbon source concentration in the production medium is known to influence PHA production significantly. [15] To determine the optimum concentration of the previously optimized carbon source for increased PHA production 4 different values were considered viz. 0.5, 1.0, 1.5 & 2 %. Fig.VII depicts an increase in PHA yield relative to increased carbon availability upto a certain concentration after which it tends to drop pertaining to excess PHA substrate in the medium. [33] Except the isolate *Bacillus subtilis* 6833 which gave maximum PHA (36.52±0.24%) at 2% carbon source concentration, all other isolates accumulated highest polymer (34 – 42%) at a carbon source concentration of 1.5% in the production medium. This was supported by results obtained by Elsayed et al., 2013; Aslam et al., 2013; Hawas et al., 2016 who have reported highest PHA yield at 1-2% optimized carbon source concentration. [23,25,37]

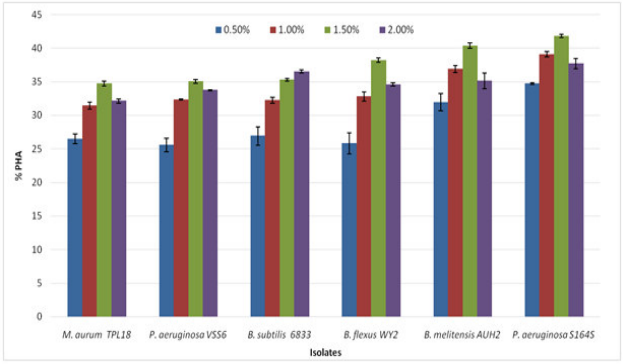


Figure VII
Effect of concentration of carbon source on PHA production

The influence of Carbon: Nitrogen ratio was determined on all the isolates with respect to PHA production and all isolates accumulated highest PHA polymer (32-42 %) at a C:N ratio of 30:1 except isolate *Microbacterium aurum* TPL18 (34.15±0.86%) which required an optimized C:N ratio of 20:1 (Fig. VIII). Above the optimized C:N ratio there was a decrease in the PHA yield which may be due to substrate inhibition phenomenon. These observations are in agreement with Nehra et al. (2012), Panigrahi and Badveli (2013), Hawas et al. (2016). [16,23,28]

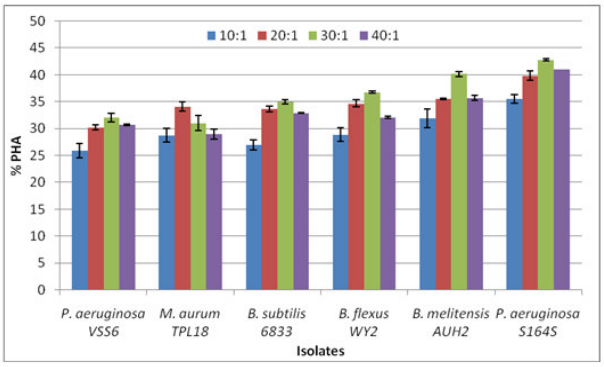


Figure VIII
Effect of carbon to nitrogen ratio on PHA production

Based on the results obtained using OFAT approach at shake flask level, Table II enlists the optimized values for each of the 8 parameters studied for all the isolates for improved production of PHA.

Table II
Summary of optimized parameters

Sr. No.	Optimized media parameters	Isolate					
		***Bacillus flexus* WY2**	***Bacillus subtilis 6833***	***Pseudomonas aeruginosa* VSS6**	***Brucella melitensis* AUH2**	***Microbacterium aurum* TPL18**	***Pseudomonas aeruginosa* S164S**
1	Temperature (°C)	37	28	37	28	28	37
2	pH	7	7	6	7	6	7
3	RPM	150	150	150	150	180	150
4	Time of incubation (h)	96	96	96	72	96	72
5	Type of nitrogen source	Yeast extract					
6	Type of carbon source	Maltodextrin	Maltodextrin	Tributyrin	Maltodextrin	Maltodextrin	Tributyrin
7	Concentration of carbon source (%)	1.5	2	1.5	1.5	1.5	1.5
8	Carbon to nitrogen ratio	30:1	30:1	30:1	30:1	20:1	30:1

Table III shows the influence of the selected parameters on the growth in terms of DCW (g/L) of isolates under study. Temperature was the first physical factor optimized and a temperature of 28°C was found to be the optimum for all the isolates. It was also observed that, more often than not higher incubation temperatures than optimum led to a decrease in biomass but supported higher PHA production (Fig.I). pH7 was found to be optimum for biomass production for the isolates *Bacillus flexus* WY2, *Bacillus subtilis 6833* , *Brucella melitensis* AUH2 and *Pseudomonas aeruginosa* S164S whereas *Pseudomonas aeruginosa* VSS6, *Microbacterium aurum* TPL18 showed maximum biomass at pH 6. Extremes of pH(5.5,8.0) were found to be adversely influencing the biomass production. Also it was observed that the same pH value was optimum for biomass as well as PHA accumulation (Fig.II).*Bacillus flexus* WY2 and *Pseudomonas aeruginosa* VSS6 accumulated highest biomass at 150 rpm while 180 rpm gave maximum biomass for *Bacillus subtilis 6833* , *Brucella melitensis* AUH2 , *Microbacterium aurum* TPL18 and *Pseudomonas aeruginosa* S164S.Higher agitation rates than optimum affected the biomass produced by the isolates which could be due to sheer forces exerted at higher rpm. [12] Isolates *Bacillus flexus* WY2, *Bacillus subtilis 6833, Pseudomonas aeruginosa* VSS6 and *Microbacterium aurum* TPL18 accumulated maximum biomass at 96 h of incubation time while *Brucella melitensis* AUH2 and *Pseudomonas aeruginosa* S164S gathered highest biomass at 72 h incubation time. Post this optimum duration the trend showed a drop which may indicate depletion of nutrients available for growth and/or accumulation of metabolites toxic for growth. [33] Apart from physical factors, medium composition affecting the growth was also studied. When original nitrogen source was replaced by the various organic and inorganic nitrogen sources it was found that the organic sources led to better growth. Also yeast extract was the best organic nitrogen source giving upto approximately 6-8 g/L of biomass for all the selected isolates. Along with nitrogen source carbon source was also found to influence growth of the selected isolates. Overall, isolates showed a comparable biomass production for most of the carbon sources screened although maltodextrin (6.03 to 7.45 g/L) and FOS (6.2 to 7.30 g/L) proved to be better amongst them for *Bacillus flexus* WY2, *Bacillus subtilis* 6833, *Brucella melitensis* AUH2 and *Microbacterium aurum* TPL18.These isolates showed poor growth (< 3 g/L) when tributyrin was used as a carbon source where as it gave maximum biomass for the other 2 isolates *Pseudomonas aeruginosa* VSS6 and *Pseudomonas aeruginosa* S164S up to 7.45 and 6.30 g/L respectively. Sucrose (4.83 to 6.86 g/L) and starch (4.85 to 7.05 g/L) were found to promote less biomass production for the selected isolates. With respect to the various concentrations of carbon source tried isolates *Bacillus flexus* WY2, *Bacillus subtilis* 6833, *Brucella melitensis* AUH2 showed an increase in biomass with increase in concentration of carbon source while *Pseudomonas aeruginosa* VSS6, *Microbacterium aurum* TPL18, *Pseudomonas aeruginosa* S164S showed a trend similar to their PHA production trend (Fig. VII). Biomass trend for all the isolates obtained with different carbon to nitrogen ratios in the medium remained similar to that obtained for increasing concentrations of carbon source.

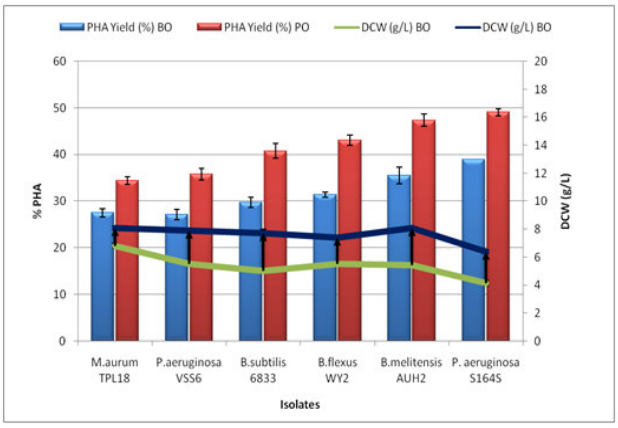
Table III
Effect of various parameters on growth of the isolates

Sr. No.	Parameter optimized		DCW (g/L)					
			***Bacillus flexus* WY2**	***Bacillus subtilis* 6833**	***Pseudomonas aeruginosa* VSS6**	***Brucella melitensis* AUH2**	***Microbacterium aurum* TPL18**	***Pseudomonas aeruginosa* S164S**
1	Temperature (°C)	20	5.26 ±0.16	4.75±0.11	5.15±0.25	5.94±0.11	6.64±0.12	3.73±0.2
		28	**5.99±0.17**	**5.34±0.09**	**6.02±0.28**	**6.18±0.23**	**7.32±0.22**	**4.32±0.13**
		37	5.52±0.18	5.00±0.16	5.51±0.14	5.41±0.18	6.82±0.15	4.15±0.18
		45	4.44±0.18	4.31±0.14	4.73±0.17	5.20±0.12	5.80±0.16	3.75±0.15
2	pH	5.5	5.44±0.07	5.80±0.17	6.09±0.15	6.33±0.09	7.53±0.12	4.08±0.13
		6.0	5.96±0.20	6.02±0.15	**6.58±0.14**	6.82±0.16	**7.99±0.14**	4.50±0.11

		7.0	**6.11±0.22**	**6.31±0.16**	6.29±0.15	**7.04±0.14**	7.67±0.14	**4.74±0.12**
		8.0	5.64±0.31	6.07±0.25	6.18±0.18	6.40±0.20	7.24±0.13	4.36±0.14
3	RPM	120	5.64±0.09	5.38±0.16	6.31±0.12	6.19±0.12	7.38±0.18	4.10±0.09
		150	**6.14±0.19**	5.83±0.13	**6.71±0.17**	6.49±0.11	7.78±0.22	4.45±0.10
		180	6.01±0.18	**5.99±0.24**	6.46±0.22	**6.85±0.06**	**7.94±0.12**	**4.62±0.10**
		210	5.84±0.06	5.58±0.24	6.11±0.27	6.64±0.17	7.74±0.12	4.25±0.19
4	Time of incubation(h)	24	2.23±0.18	2.56±0.14	2.73±0.13	3.04±0.14	3.15±0.22	1.83±0.15
		48	5.88±0.19	6.01±0.25	6.01±0.16	6.81±0.16	7.51±0.21	4.71±0.24
		72	7.10±0.16	7.08±0.23	7.20±0.15	**7.69±0.18**	8.02±0.19	**5.51±0.29**
		96	**7.83±0.19**	**7.97±0.21**	**8.00±0.09**	7.29±0.17	**8.87±0.11**	5.17±0.12
		120	6.97±0.23	7.06±0.18	6.96±0.15	-	7.66±0.26	-
5	Nitrogen source	Yeast extract	**7.47±0.33**	**7.29±0.26**	**7.96±0.23**	**7.45±0.20**	**7.98±0.25**	**5.81±0.27**
		Peptone	7.08±0.33	6.85±0.20	7.66±0.24	6.99±0.26	7.76±0.23	5.44±0.21
		Tryptone	6.16±0.27	6.13±0.24	7.06±0.24	6.69±0.21	7.38±0.18	5.17±0.11
		Ammonium Chloride	5.59±0.25	5.13±0.24	6.52±0.30	5.87±0.20	6.80±0.16	4.96±0.15
		Ammonium Sulphate	5.54±0.23	5.08±0.22	6.30±0.26	5.71±0.22	6.26±0.22	4.74±0.21
		Ammonium acetate	6.69±0.27	6.78±0.24	6.29±0.17	6.77±0.23	7.44±0.16	4.59±0.25
6	Carbon source	Glucose	6.20±0.17	6.65±0.28	6.52±0.10	6.78±0.10	7.17±0.08	5.91±0.29
		Sucrose	5.83±0.28	6.11±0.09	6.22±0.11	6.06±0.14	6.86±0.16	4.83±0.13
		Lactose	6.86±0.10	7.00±0.12	6.97±0.08	7.10±0.10	7.27±0.22	5.95±0.24
		Glycerol	7.05±0.10	6.27±0.09	6.91±0.13	6.99±0.08	7.22±0.14	4.76±0.15
		Mannitol	7.07±0.26	6.87±0.13	7.13±0.12	7.27±0.17	7.38±0.12	5.77±0.22
		Starch	6.02±0.19	6.69±0.20	6.41±0.13	6.58±0.10	7.05±0.13	4.85±0.25
		Fructooligosaccharide	**7.28±0.09**	**7.04±0.12**	7.16±0.08	**7.16±0.11**	7.30±0.22	6.20±0.28
		Maltodextrin	7.14±0.14	7.07±0.07	7.22±0.05	7.04±0.16	**7.45±0.07**	6.03±0.14
		Tributyrin	2.30±0.16	2.77±0.18	**7.45±0.06**	2.93±0.23	2.53±0.16	**6.30±0.04**
7	Concentration of carbon source (%)	0.5	4.73±0.21	4.29±0.16	4.89±0.18	4.83±0.10	4.24±0.13	4.63±0.09
		1	6.67±0.16	6.63±0.10	6.15±0.10	7.10±0.08	5.96±0.13	6.11±0.07
		1.5	7.07±0.18	7.18±0.10	**6.95±0.12**	7.19±0.17	**6.33±0.10**	**6.77±0.12**
		2	**7.60±0.12**	**7.22±0.18**	6.57±0.11	**8.08±0.06**	6.10±0.09	6.32±0.06
8	Carbon to nitrogen ratio	10:1	4.58±0.19	4.37±0.17	5.01±0.18	4.72±0.08	4.34±0.08	4.46±0.11
		20:1	6.53±0.17	7.01±0.22	6.91±0.16	7.26±0.08	6.01±0.08	6.14±0.06
		30:1	7.35±0.08	7.45±0.07	**7.46±0.06**	7.37±0.11	**6.21±0.17**	**6.73±0.16**
		40:1	**7.93±0.08**	**7.51±0.17**	7.16±0.07	**7.84±0.13**	6.04±0.12	6.44±0.08

Key: The highlighted cells indicate results of the optimum parameters for each isolate

Post optimization, all the isolates showed significant improvement (25-40%) in PHA production which can be attributed to the optimization of parameters critical to PHA accumulation. Also there was a noticeable increase in the biomass obtained for all the isolates as compared to the original M9 growth medium and culture conditions (Figure IX).



Key:-BO-Before optimization , PO-Post optimization

Figure IX
Summary of growth (DCW) and PHA yield

CONCLUSION

Fermentation conditions are known to greatly influence productivity of secondary metabolites.Hence the present study was undertaken to optimize the shake flask parameters so as to obtain maximum PHA yield by the selected isolates. Amongst the 6 bacterial isolates identified 2 bacterial species namely *Brucella melitensis* AUH2 and *Microbacterium aurum* TPL18 were found to be novel as PHA producers. Optimization of media parameters and culture conditions resulted in an appreciable increase in PHA production along with biomass. Total of 8 parameters were optimized at OFAT level, from which agitation rate, concentration of carbon source and carbon to nitrogen ratio in the production medium were found to be playing significant role in PHA production.These parameters can be further optimized by response surface methodology so as to improve PHA yield. *Bacillus flexus* WY2, *Brucella melitensis* AUH2 and *Pseudomonas aeruginosa* S164S gave maximum PHA yield after optimization and hence could be promising for further optimization.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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