Summary

Environmental concerns have spurred interest towards the microbial production of various biopolymers including polyhydroxyalkanoates (PHA). PHAs are produced by bacteria in response to stress. Oil-contaminated soils are considered stressed sites. The presence of excess carbon with limited nitrogen makes these sites a potential source for isolating PHA producing bacterial strains. Realizing this, the present investigation “Enhanced synthesis of polyhydroxyalkanoates (PHA) in bacterial strains isolated from hydrocarbon-contaminated sites” from oil contaminated soils was carried out.

In the present investigation, two methods, a phenotypic method i.e. Nile Blue A colony staining and a genotypic method i.e. Polymerase Chain Reaction for amplification of the phacC gene were used to screen for PHA producing bacterial strains. A total of two hundred and sixty two bacterial strains isolated from 14 distant oil-contaminated sites were screened for PHA production. The screening results showed that 73 bacterial strains were positive with the genotypic method and 66 with the phenotypic method. A total of 41 bacterial strains were positive with both the methods. The quantification of the PHA in these bacterial strains was done by gas chromatography. Among these 41 strains, 3 bacterial strains, *Brochothrix thermosphacta* TERI 5001, *Sphingobacterium* sp. TERI 13006 and *Yokonella regensburgei* TERI 2003, which have not been described for PHA production, also showed the production of PHA.

Amongst the 41 bacterial strains, two strains, *Brochothrix thermosphacta* TERI 5001 and *Pseudomonas aeruginosa* TERI 13012 were selected for further studies on the basis of higher PHA content.

Initially attempts were made to optimize the physiological parameters for maximum growth of *B. thermosphacta* TERI 5001 and *P. aeruginosa* TERI 13012. Results showed that *B. thermosphacta* TERI 5001 strain grew optimally at a temperature of 30°C, pH of 6.5 and agitation at 150 rpm and *P. aeruginosa* TERI 13012 grew optimally at temperature of 35 °C, a pH of 7.0 and agitation at 150 rpm.

To enhance the production of PHA by these 2 bacterial strains, two approaches “one-at-a-time” method and “Response Surface Methodology” were followed. Parametric optimization by “one-at-a-time” method were done for the both the bacterial strains. The results showed that under the conditions of fructose 1.5 %, NH₄Cl 0.01 %, KCl 0.1
% and inoculum density of 5 %, 35 % PHA wt/wt of CDW) was produced in 120 h by 
*B. thermosphacta* TERI 5001. This is the first study where the conditions for PHA 
production by a *B. thermosphacta* strain are studied.

*P. aeruginosa* TERI 13012 produced 49 % of PHA in 96 h under conditions of 1.5 % 
oleic acid, 0.05 % (NH₄)₂HPO₄, 0.1 % MgCl₂ and 5 % inoculum density.

Based on the results of “one-at-a-time” method *P. aeruginosa* TERI 13012 was 
chosen for optimization with Response surface methodology, since this strain could 
accumulate 49 % PHA in comparison to 35 % by *B. thermosphacta* TERI 5001. 
Before doing response surface methodology, 2-level factorial design was done to 
screen for variables which were affecting PHA production significantly.

On the basis of the results of 2-level factorial design, oleic acid, (NH₄)₂HPO₄, K₂HPO₄ 
and KH₂PO₄ were chosen for response surface methodology wherein the interaction 
of these variables were examined for further enhancement of PHA production by *P. 
aeruginosa* TERI 13012. The optimum conditions for maximum PHA production in 
MSM medium were oleic acid 0.75 %, (NH₄)₂HPO₄ 0.04 %, 0.2 % KH₂PO₄ and 0.4 % 
K₂HPO₄. These conditions resulted in 58 % of PHA in 96 h.

From the results of the RSM experiment, it was seen that the effect of oleic acid, 
(NH₄)₂HPO₄ and KH₂PO₄ was negative for PHA production and the effect of K₂HPO₄ 
was positive. Significant interactions were noted between the nitrogen source 
(NH₄)₂HPO₄ and the phosphate source KH₂PO₄. Also the linear, quadratic and 
interactive effects of (NH₄)₂HPO₄ were more significant than other variables 
highlighting its role in PHA accumulation within cells. The optimum C/N ratio found 
from our study was 19 (mol/mol).

Time course experiment was done with the conditions optimized by RSM. The results 
showed that PHA production was initiated at 48 h and reached a maximum of 60 % 
of CDW at 120 h. The percentage of PHA within cells remained almost constant from 
72 h to 168 h. Intracellular consumption of PHA was not seen with these conditions.

Scale up of PHA production in 150 liter bioreactor in the medium optimized by 
response surface methodology resulted in the production of 54 % PHA at 96 h and 
the CDW obtained was 3.9 g/l which was three times higher than what was obtained 
in shake flask experiments. The final yield of PHA was 0.26 g/g of oleic acid.

PHA was extracted from the medium by solvent extraction. About 1.5 g of PHA was 
extracted per litre. The PHA was yellowish in color and sticky in nature. The PHA 
consisted of 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic 
acid, 3-hydroxoydecanoic acid and 3-hydroxytetradecanoic acid along with 3- 
hydroxydodecanoic acid and 3-hydroxytetradecanoic acid as monomeric units, which 
was confirmed by NMR studies. Unusual monomeric units were not seen in the PHA
in spite of getting *B. thermosphacta* TERI 5001, *Sphingobacterium* sp. TERI 13006 and *Yokonella regensburgei* TERI 2003 which are not known for PHA production.

The *phaC* gene of these three bacterial strains was sequenced. The phylogenetic analysis revealed that bacterial isolates from our study formed a separate cluster and was homologous to class II PHA synthase, found in different species of *Pseudomonas*. The cluster formed from this study showed closest similarity to the cluster formed by *Pseudomonas* sp. isolated from oil-contaminated sites of China.

An attempt was also made to relate the number of PHA producers isolated from a site to the ecological factors. On the basis of the results 99 % variance within the sites could be explained by history of contamination and annual rainfall.