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Full Length Research Paper

Screening of root nodule bacteria for the production of polyhydroxyalkanoate (PHA) and the study of parameters influencing the PHA accumulation

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Twelve polyhydroxyalkanoate (PHA) producing microbes were isolated from root nodules of 8 leguminous plants belonging to two phyla: Proteobacteria and Firmicutes. One of the isolate VK-12 of genus *Burkholderia* showed the highest PHA accumulation (42% wt/wt) as compared to other isolates in mineral medium. The effect of different cultural and growth conditions were studied on isolate VK-12 in shake flasks for highest PHA accumulation. VK-12 showed highest PHA accumulation in sucrose and ammonium sulphate amongst other carbon and nitrogen sources tested in the medium. The medium containing sucrose and ammonium sulphate having C: N ratio of 39.72 gave the highest PHA accumulation. The optimum pH, temperature, inoculum concentration and incubation time for highest PHA accumulation were 7.0, 30°C, 10% and 48 h respectively. An overall increase in PHA accumulation from 42 to 63% wt/wt was obtained under optimised conditions. The PHA was characterised using gas chromatography-mass spectrometry (GC-MS). All the isolates produced poly (3-hydroxybutyrate) (PHB) except VK-9, which produced polymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate).

Key words: Polyhydroxyalkanoate (PHA), PHB-co-HV, root nodule bacteria, carbon sources, C: N ratio, characterization of PHA.

INTRODUCTION

Decades have been invested on extensive research to develop biodegradable polymers as a substitute for petrochemical based polymers due to their eco-friendly nature. Polyhydroxyalkonoates (PHA) are polyesters of various R-hydroxyalkanoates and are considered as a good alternative amongst other biodegradable polymers developed, due to their biodegradability, biocompatibility, use of renewable resources as raw material, plastic and elastomeric material properties similar to petrochemical based polymers (Lee, 1996a; Ojumu et al., 2004). PHA's are accumulated as intracellular inclusion bodies by many Gram-positive and Gram-negative bacteria to levels as high as 90% of dry cell weight when carbon source is in excess but other nutrient supply (O, P, N and S) are in limiting condition (Anderson and Dawes, 1990; Lee, 1996b). A large number of PHA's and its copolymers have been exploited as bioplastics, biomedical applications such as drug delivery (Gursel et al., 2002; Sendil et al., 1999) and tissue engineering (Chen and Wu, 2005; Misra et al., 2006).

Various researchers have isolated the promising micro-organisms from different environment such as municipal sewage sludge (Reddy et al., 2008), marine microbial mats (López-Cortés et al., 2008) and marine environments (Arun et al., 2009). The root nodules of leguminous plants can be regarded as a good ecosystem for the isolation of potential PHA producing isolates as plants interact with abundant and diverse range of bacteria present in the soil. In the present work, isolation and screening of PHA producing bacteria has been carried out from the root nodules of leguminous plants. The major problem associated with the industrial poly β-

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Abbreviations: PHA, Polyhydroxyalkanoate; **GC-MS,** gas chromatography-mass spectrometry; **PHB,** poly (3-hydroxybutyrate).

hydroxybutyrate (PHB) production is its cost of production that could be minimised by cultural optimization studies. Hence, different cultural and environmental conditions were tested to analyze their effect on PHA production by the higher PHA producing isolate.

MATERIALS AND METHODS

Isolation and identification of PHA producing bacteria

Plants of *Glycine max, Vigna sinensis, Vigna umbellate, Arachis hypogaea,* and *Vigna mungo*, grown and maintained in the fields of Birsa Agricultural University, Ranchi, India, and *Acacia catechu, Mimosa pudica and Trifolium repens* grown in the campus of Birla Institute of Technology (BIT), Mesra, Ranchi, during the winter (November to December) of 2009 were uprooted, root nodules (4 to 6 per plant) were collected and stored in air tight plastic containers. Bacterial isolation was carried from nodules according to the method of Zakhia et al. (2006).

The bacterial isolates were further evaluated for PHA accumulation by incubating them for sufficient time (72 h) at 28°C on YEMA plates. PHA accumulation of the bacteria was first checked by Sudan Black B staining (Burdon, 1946) and then confirmed with Nile Blue A staining (Ostle and Holt, 1982).

PHA producing isolates were subsequently identified based on 16S rRNA sequencing. Genomic DNA was extracted and purified according to standard protocols (Sambrook and Russell, 2001) and its purity was assessed by measuring the absorbance at 260 and 280 nm (A260/A280). The 16S rRNA was PCR amplified using the following universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' ACGGCTACCTTGTTACGACTT-3'). The amplified samples were sent to Xcelris laboratories, Ahmadabad, India, for sequencing service. Chromatograms, thus received were analyzed while contigs were assembled manually using Chromas Pro version 1.5 (Technelysium Ptv, Ltd, Tewantin, Australia). The 16S rRNA gene sequences were compared with other known rRNA gene sequences from Ribosome Database Project (RDP) (http://rdp.cme.msu.edu/). Seq match programme of RDP was used to search the most similar sequences in the databases (Cole et al., 2007, 2009).

PHA production

Growth medium and culture conditions

Pre inoculum of all the selected cultures was prepared in mineral medium (Borah et al., 2002) with glucose (2% w/v) as carbon source. PHA production was carried out in 100 ml mineral medium in a 500 ml Erlenmeyer flask. A 5% pre-inoculum culture was inoculated into the 100 ml production medium described earlier and incubated in an orbital shaker at 150 rpm and 30°C for 48 h. Using the same method, all 12 isolates were incubated for 48 h (30°C). After incubation, the broth was centrifuged at 8,000 rpm for 10 min; cells collected were washed with distilled water to remove the remaining media constituents and cells were lyophilized for the extraction of PHA. To estimate dry cell weight, 10 ml of the broth was centrifuged separately and cells were dried to a constant weight at 60°C for 48 h.

Optimization studies

VK-12 isolate was further used for the optimization studies in shake flask. The media used for these experiments were the same as earlier described.

Effect of different carbon and nitrogen sources and C: N ratio

Glucose and ammonium sulphate of the mineral medium was replaced with different carbon sources (sucrose, mannitol, glucose, lactose, xylose, maltose and starch) and nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulfate, tryptone, yeast extract, peptone, beef extract, malt extract). The amount of carbon and nitrogen compound added was calculated to give the same concentration as the original source. The C:N ratio varied from 9.93 to 79.44 by keeping sucrose concentration at 20 gL^{-1} and varying ammonium sulphate concentration from 2.0 to 0.5 gL^{-1} in the mineral medium. Other cultural conditions were kept constant. Samples were analysed for cell growth and PHA accumulation.

Effect of different pH, temperature, inoculum concentration and incubation time

The effect of different pH, temperature, inoculum concentration and incubation time was determined by growing the isolate in mineral media having sucrose and ammonium sulphate with C: N ratio of 39.72. The pH of the medium varied in the range 6 to 9, temperature in the range of 25 to 50°C, cell concentration of 12 h old inoculum in the range of 1×10^4 to $1x\ 10^5$ cells in 100ml medium, incubation time varied from 0 to 60 h and the fermentation was carried as stated before. Samples were analysed for cell growth and PHA accumulation.

Quantification and characterization of PHA

The PHA content of the isolates was quantified using UV-spectrophotometer at 208 nm according to the method of Karr et al. (1983). Standard curve was prepared with poly β-hydroxybutyrate (PHB) (Sigma-Aldrich Co. Ltd). For the extraction of PHA, the lyophilized cells were treated with hypochlorite solution (4 to 6% w/v active chlorine). At this concentration, the cell will lyses without degradation of PHA. Then the cells were subjected to homogenisation by ultrasonic treatment for 5 min for complete cell lyses. The homogenate was further kept at 37°C for 30 min in orbital shaker. The lysed cell mass was centrifuged at 10,000 rpm for 15 min, the pellet obtained was washed with sterile distilled water and then with cold diethyl ether. The residue obtained was finally dried and subjected to chloroform extraction at 50°C for 24 h. PHA present was concentrated by rotary vacuum evaporation and precipitation using 10 volumes of ice-cold methanol. The precipitate obtained was centrifuged and air-dried.

GC-MS analysis

Methyl esters of the extracted PHA were prepared (Braunegg et al., 1978). The analysis was performed using Perkin Elmer Clarus 500 GC/MS. Elite 17 MS column (30 m-length, 0.25 mm-internal diameter, and 0.25 µm-film thickness) was used. The sample (1 µl) in chloroform was injected with helium (1ml/ min) as the carrier gas. The injector temperature was 230°C and the column temperature was increased from 80 to 150°C at 10°C min^{-1}.

RESULTS

Isolation and identification of PHA producing bacteria

Dilution of root nodule extract on YEMA plate gave rise to a large number of colonies with a great variation in colony

Table 1. List of root nodule bacteria isolated and screened for PHA production.

Host plant	Number of colony screened for PHA production [a]	Number of PHA producing isolates [b]
Acacia catechu	8	6
Trifolium repens	5	1
Mimosa pudica	7	1
Glycine max	7	-
Vigna sinensis	4	2
Vigna umbellata	9	1
Arachis hypogaea	5	-
Vigna mungo	9	1

[a]No of colonies picked from second plate for screening; [b] based on Sudan black B and confirmation with Nile blue A staining.

Table 2. PHA yield and its monomeric composition obtained from isolates.

Isolate	Dry cell mass (g L^{-1}) *	PHA (g L^{-1}) *[a]	%PHA* [b]	PHA content
Vk-4	2.43±0.04	0.51±0.02	21±0.17	PHB
VK-5	1.73±0.03	0.42±0.03	24±0.23	PHB
VK-6	2.03±0.31	0.45±0.03	22±0.09	PHB
VK-7	2.53±0.17	0.62±0.03	25±0.03	PHB
VK-8	6.24±0.21	2.07± 0.12	33±0.21	PHB
VK-9	6.66±0.071	2.60± 0.03	39±0.15	P3HBco HV
VK-10	2.11±0.05	0.39±0.04	18±0.11	PHB
VK-11	6.22±0.12	1.85±0.08	30±0.18	PHB
VK-12	6.35±0.11	2.83±0.03	42±0.06	PHB
VK-13	6.61±0.03	2.41±0.05	36±0.06	PHB
VK-14	4.08±0.11	1.42±0.03	30±0.13	PHB
VK-15	3.70± 0.02	1.48±0.04	40±0.19	PHB

*Values are the means of triplicate measurements with standard deviations; [a]PHA content in the medium; [b]PHA accumulation based on cell dry weight.

morphology, colour and size. Out of these, a total of 54 bacterial isolates were screened for PHA producing potentials. Twelve PHA producing isolate were obtained from the root nodules (Table 1). These isolates, named VK-4 through VK-15, were screened on the basis of results of staining with Sudan black B and Nile blue-A stains.

The 16S rRNA sequence analysis of the isolates showed that they belonged to seven genera (*Bacillus* (VK-4 through VK-7), *Ensifer* (VK-8), *Rhizobium* (VK-9), *Pseudomonas* (VK-10), *Burkholderia (*VK-11 through VK-13), *Delftia* (VK-14) and *Cupriavidus* (VK-15)). The 16S rDNA sequences of various isolates have been deposited in EMBL database under accession numbers FR853803 through FR853814 for isolates VK4-VK-15.

PHA production

In shake flask experiment, the production of PHA by the 12 isolates ranged between 0.4 and 2.8 gL^{-1} when incubated for 48 h at 30°C. The percentage PHA productivity was highest for VK-12 (42% wt/wt) based on dry cell mass (Table 2).

Optimization studies

Effect of different carbon and nitrogen sources and C: N ratio

The isolate was able to utilize all seven carbon sources for growth and PHA accumulation; it produced maximum PHA with sucrose followed by mannitol, glucose, lactose, xylose, maltose and starch. The amount of PHA produced using sucrose as sole carbon source was 3.56 g L^{-1}, corresponding to 50% of cell dry weight (Figure 1). However, starch was least suitable for the growth and PHA production. The influence of different nitrogen sources on the growth and PHB production by the isolate

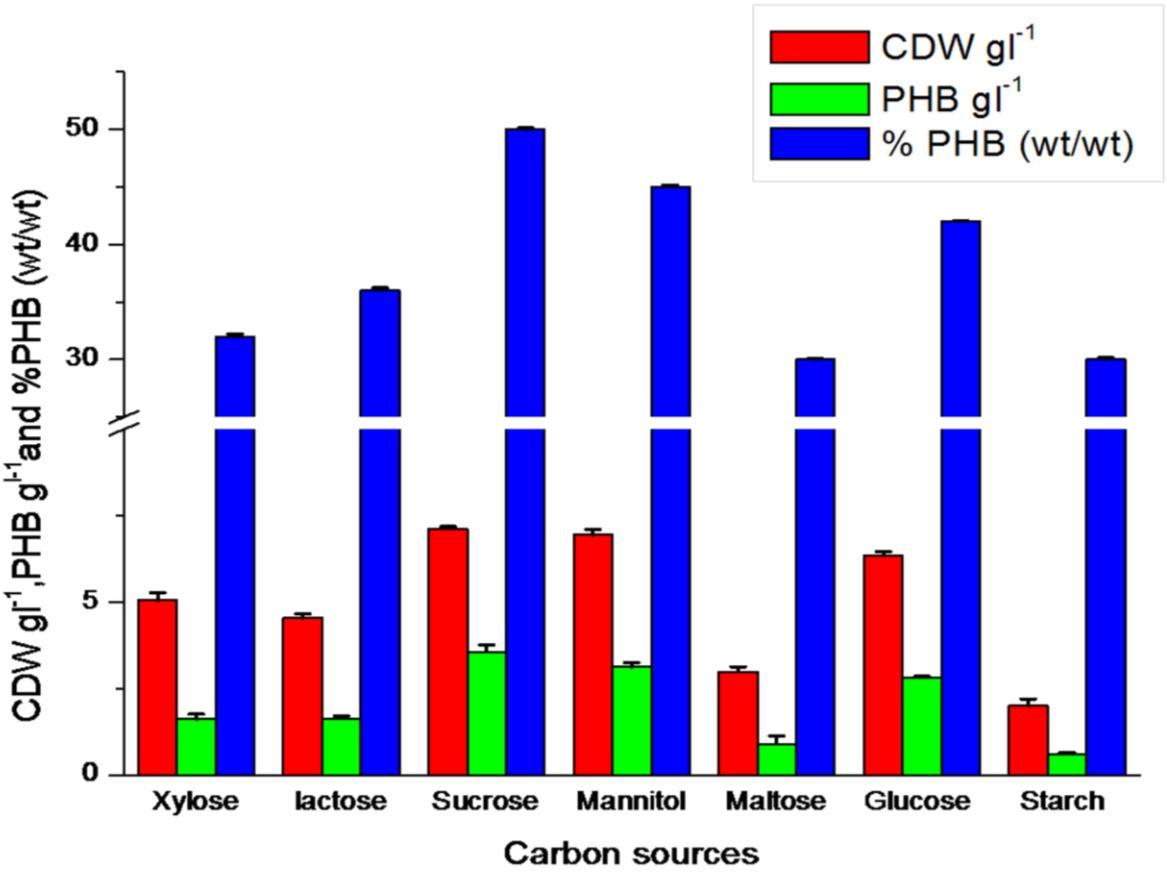


Figure 1. Effect of different carbon sources on growth and PHB production of isolate VK-12.CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt) = PHB accumulation based on cell dry weight.

in media containing sucrose as a carbon sources have shown that yeast extract and ammonium sulphate gave the highest biomass yield though percentage PHA accumulation was found to be highest for ammonium sulphate as compared to yeast extract. The maximum PHA accumulation was obtained in case of ammonium sulphate followed by tryptone, yeast extract, peptone, beef extract, malt extract, ammonium chloride and ammonium nitrate (Figure 2). Maximum PHA concentrations were obtained with sucrose and ammonium sulphate; hence, both have been used in all subsequent work. Optimisation studies with varying C: N ratio show that highest biomass yield was obtained at C:N ratio of 13.24 while highest PHA accumulation was obtained at C:N ratio of 39.72 (Figure 3).

Effect of different pH, temperature, inoculum concentration and incubation time

The optimum growth of isolate VK-12 was observed at pH 7.0 and also at this pH, the maximum accumulation of PHB by this isolate was obtained (Figure 4). Although the culture grew at lower and higher pH values, a significant decrease in PHB accumulation was observed.

It is known that temperature is one of the most critical parameters that have to be controlled in the bioprocess. It is obvious from the results (Figure 5) that 30°C was generally more favourable for PHA production. However, the temperature above 30°C resulted in a sharp decrease in PHA accumulation. The results indicate that the use of 5×10^4 cells in 100 ml medium as inoculation gave the highest PHA accumulation (Figure 6). Higher or lower inoculum concentration resulted in a significant decrease in PHA accumulation.

Studies on effect of incubation time show that the PHB production increased by increasing its time up to 48 h. Accumulation of the polymer begins in the late log phase of growth and becomes maximum during the stationary phase of growth after PHA decreased in time dependent manner, thus, 48 h was selected as the optimum incubation time (Figure 7).

PHA characterisation

GC-MS analysis

The polymers extracted from 12 isolates were analyzed by GC-MS. Eleven isolates produced PHB as observed

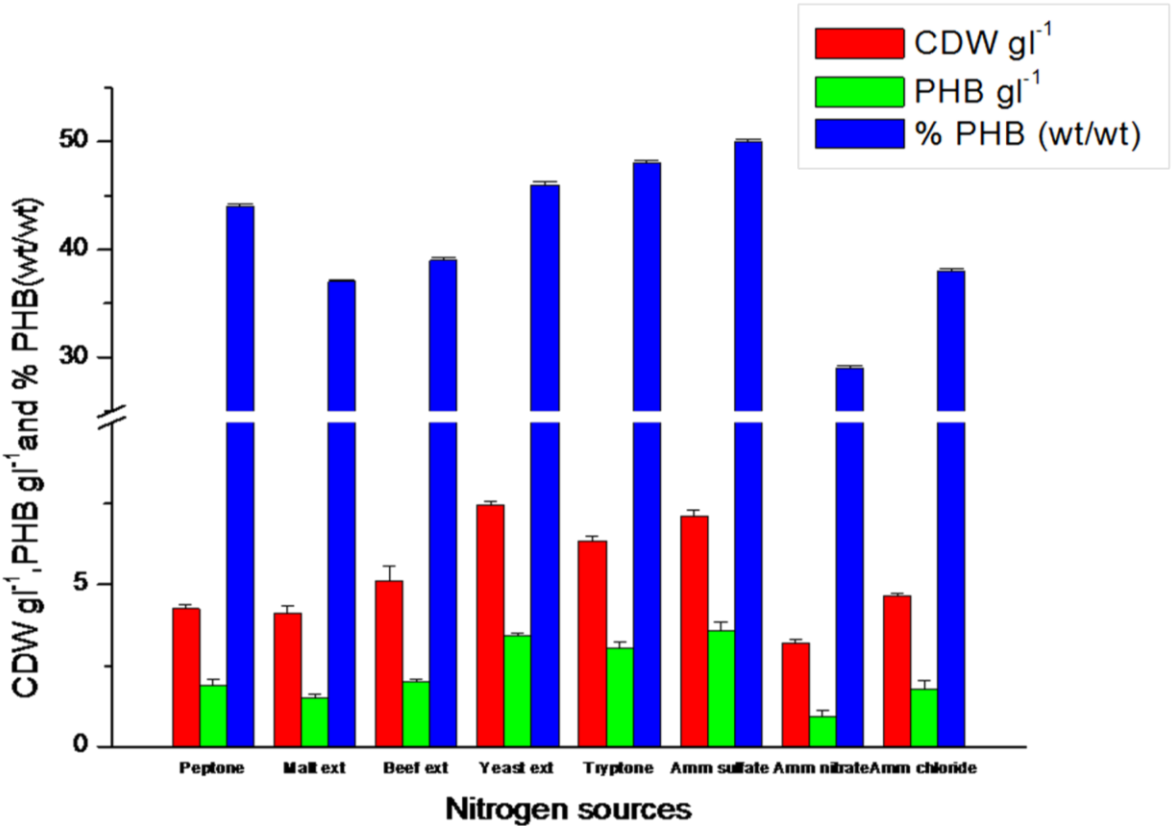


Figure 2. Effect of different nitrogen sources on growth and PHB production of isolate VK-12 CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt) = PHB accumulation based on cell dry weight.

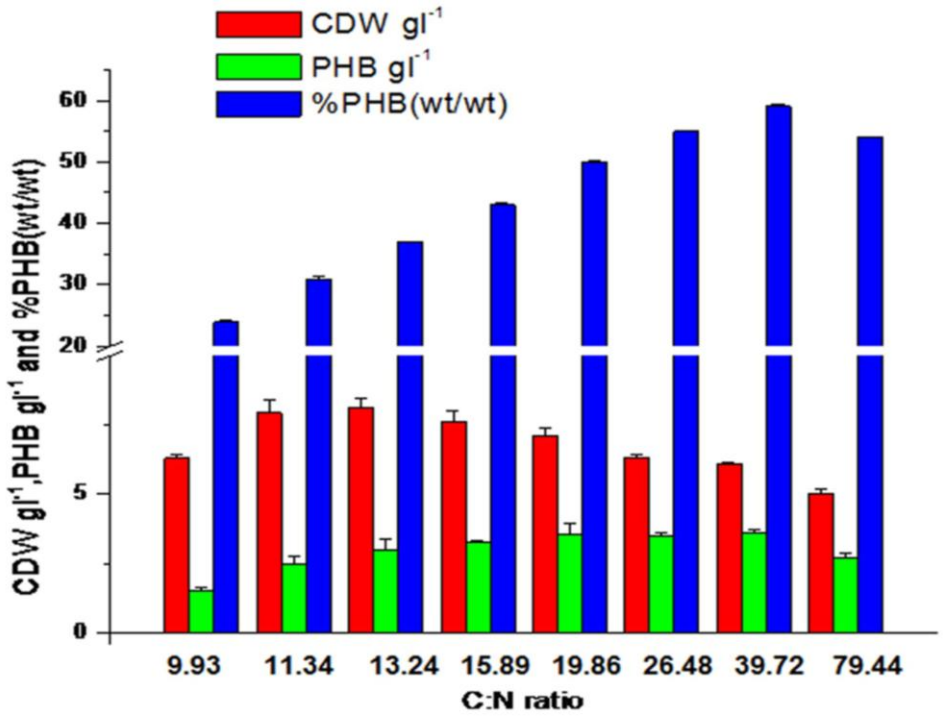


Figure 3. Effect of different C: N ratio on growth and PHB production of isolate VK-12. CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt)= PHB accumulation based on cell dry weight.

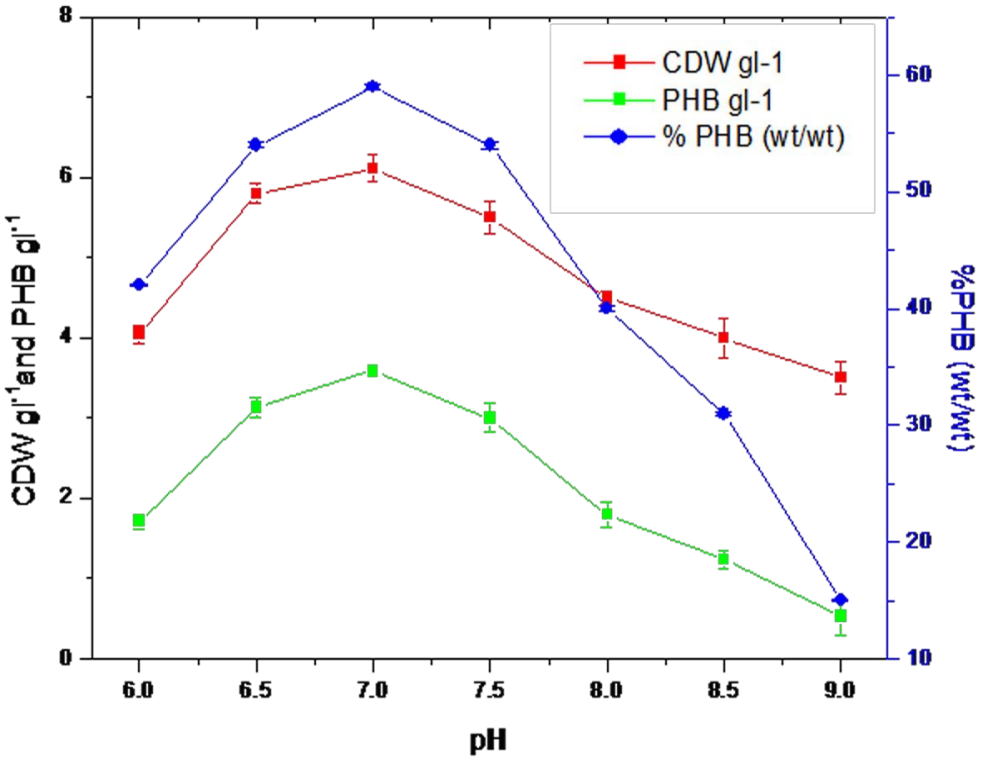


Figure 4. Effect of different pH on PHB production by isolate VK-12. CDW gl^{-1}= Cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt)= PHB accumulation based on cell dry weight.

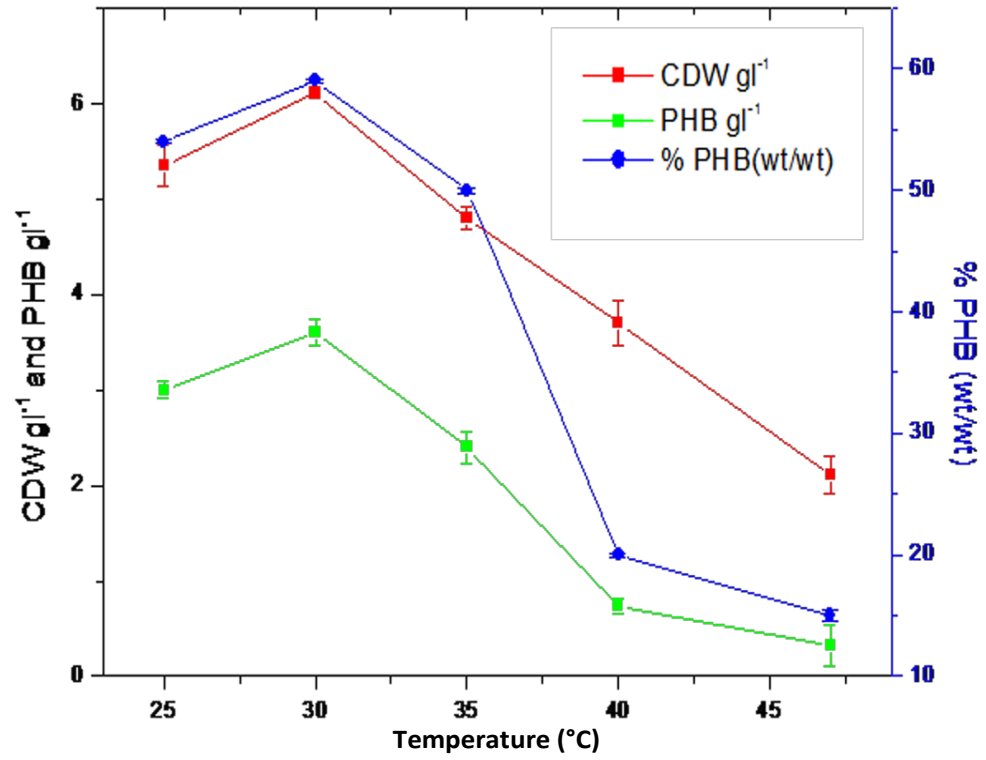


Figure 5. Effect of different temperature on PHB production by isolate VK-12. CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt) = PHB accumulation based on cell dry weight.

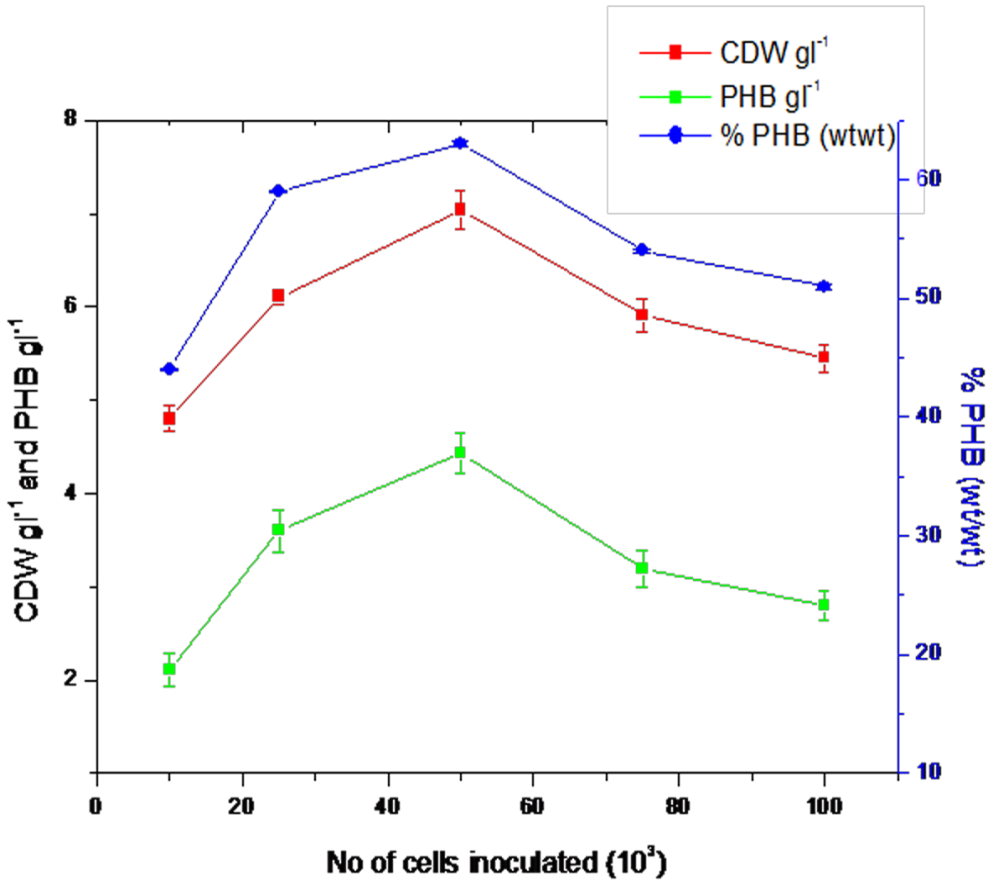


Figure 6. Effect of different inoculum concentration/100 ml medium on PHB production by isolate VK-12. CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt) = PHB accumulation based on cell dry weight.

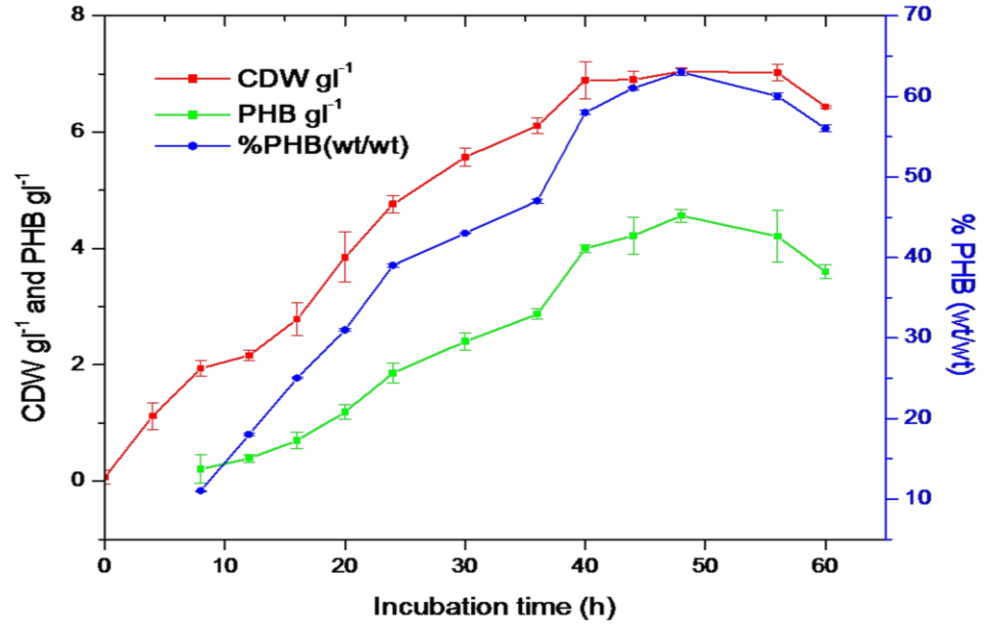


Figure 7. Effect of incubation time on biomass yield and PHB production by isolate VK-12. CDW gl^{-1}= cell dry weight, PHB gl^{-1}= PHB content in the medium, %PHB (wt/wt) = PHB accumulation based on cell dry weight.

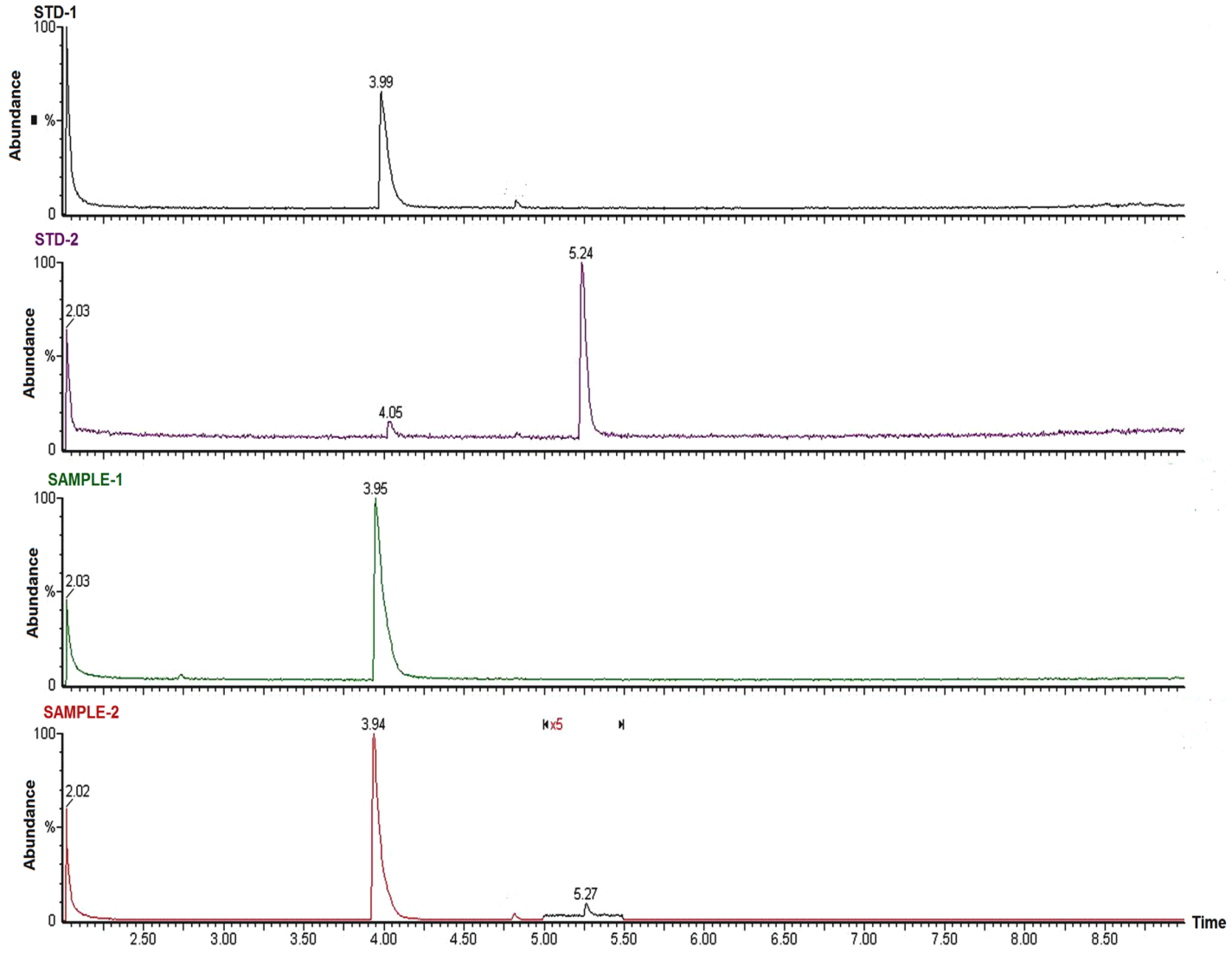


Figure 8. The total ion current chromatogram (TIC) of the methanolysis product of the polymer isolated. STD1= standard methyl ester of 3-hydroxybutyrate, STD2= standard methyl ester of 3-hydroxy valerate, sample1= polymer extracted from VK-8 and Sample2= polymer extracted from VK-9. Peak was magnified 5 times.

by a peak of methyl ester of 3-hydroxybutyrate (3.99) and only VK-9 gave an additional peak at 5.27 which corresponds to methyl ester of 3-hydroxyvalerate. The mass spectrum and retention time of these peaks also matched that of the standard sample of the methyl ester of 3-hydroxybutyrate (Sigma–Aldrich) and methyl ester of 3-hydroxyvalerate (Sigma-Aldrich) (Figures 8 and 9). The chromatogram peak with retention time of 3.99 min also matched the mass spectrum from the MS library (NIST 2005), for the methyl ester of 3-hydroxybutyrate.

DISCUSSION

Bacteria are diverse and abundant in soils. Therefore, plants are constantly involved in interactions with a wide range of bacteria, and a number of plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and inside of the plant tissues endophytes. Our research goals include the isolation of PHA producing microbes from root nodules of leguminous plants, identifying the PHA producing ability, quantifying and characterising the PHA produced by these isolates. In this study, we isolated 12 potential PHA producing strains from root nodules of *A. catechu*, *T. repens*, *M. pudica*, *V. sinensis, V. umbellate and V. mungo* (Table 2). Considering 16S rRNA gene sequencing results, these strains commonly belong to two phyla: Proteobacteria and Firmicutes with seven genera (*Bacillus*, *Ensifer*, *Rhizobium*, *Pseudomonas*, *Burkholderia*, *Delftia* and *Cupriavidus*). Half of the PHA producing isolates were obtained from *A. catechu* and

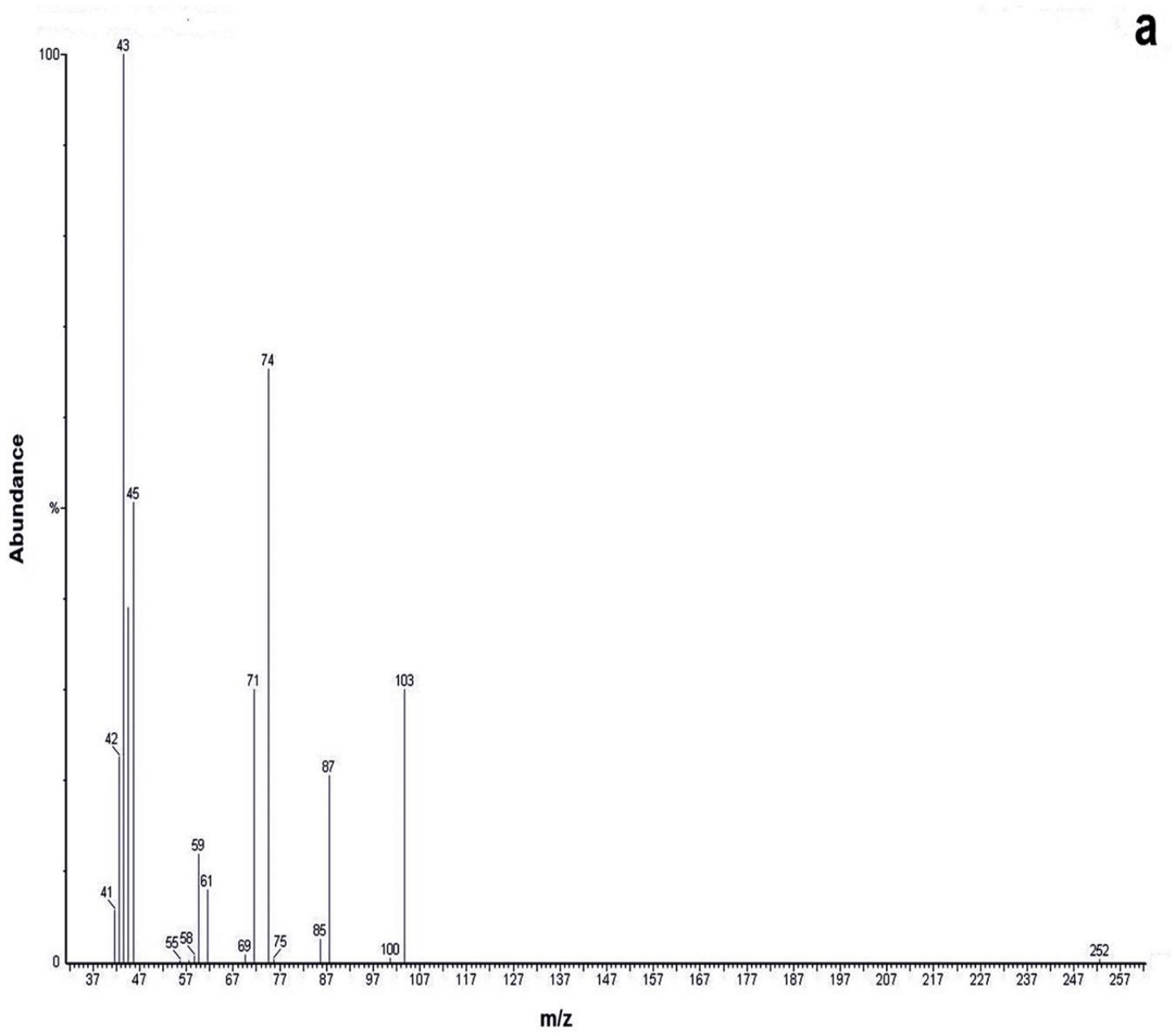
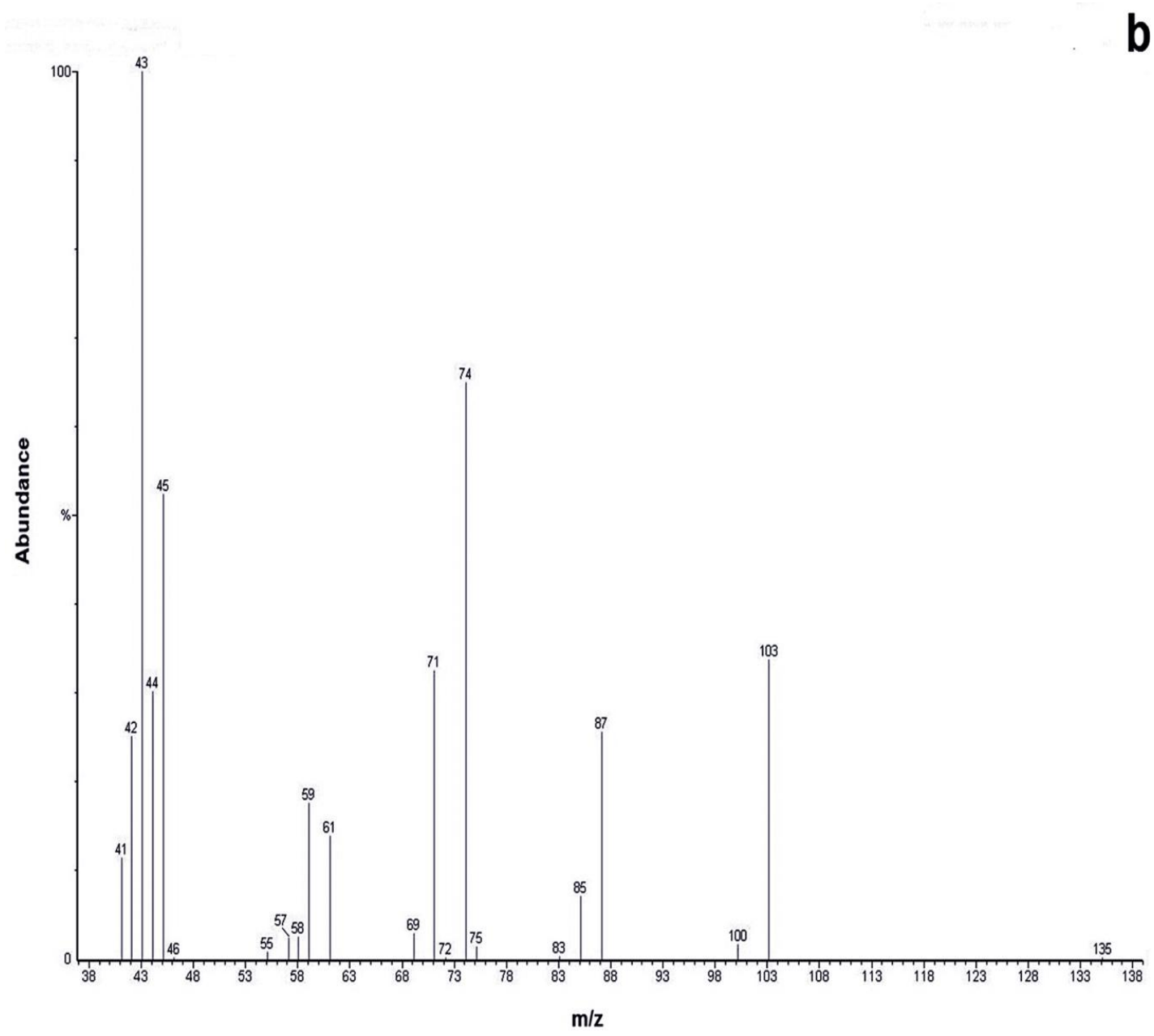


Figure 9. Mass spectra of (a) standard methyl ester of 3-hydroxy butyrate (3HB), (b) methyl ester of polymer extracted from VK-8, (c) standard methyl ester of 3-hydroxy valerate (3HV) and (d) methyl ester of polymer extracted from VK-9.

belonged to genus *Bacillus*, *Ensifer*, *Rhizobium, Pseudomonas* and *Burkholderia*. Among all the isolates, VK-12 emerged as the highest PHA producer (42% wt/wt) based on dry cell weight (Table 2) and it belonged to genus *Burkholderia*. *Burkholderia* sp. is known for one of the most metabolically versatile bacterium (Stanier et al., 1966), therefore, it was able to utilise all tested carbon source for growth and PHA production; however, the PHA accumulation was found to be highest with sucrose as carbon source as compared to other carbon sources tested. Lee and Yim (1995) have reported similar kind of results in their study on *Pseudomonas cepacia* KYG-505 (KCCM 10004) that sucrose as a carbon source gave the highest cell growth and PHA accumulation as compared to fructose, glucose and maltose. In this study, it was observed that the highest biomass yield and PHA accumulation was obtained using ammonium sulphate as nitrogen source compared to other nitrogen sources tested. However, Borah et al. (2002) reported that organic nitrogen sources increased the PHB accumulation rather than inorganic nitrogen sources based on the study on *Bacillus mycoides*. In contrast to this, El-



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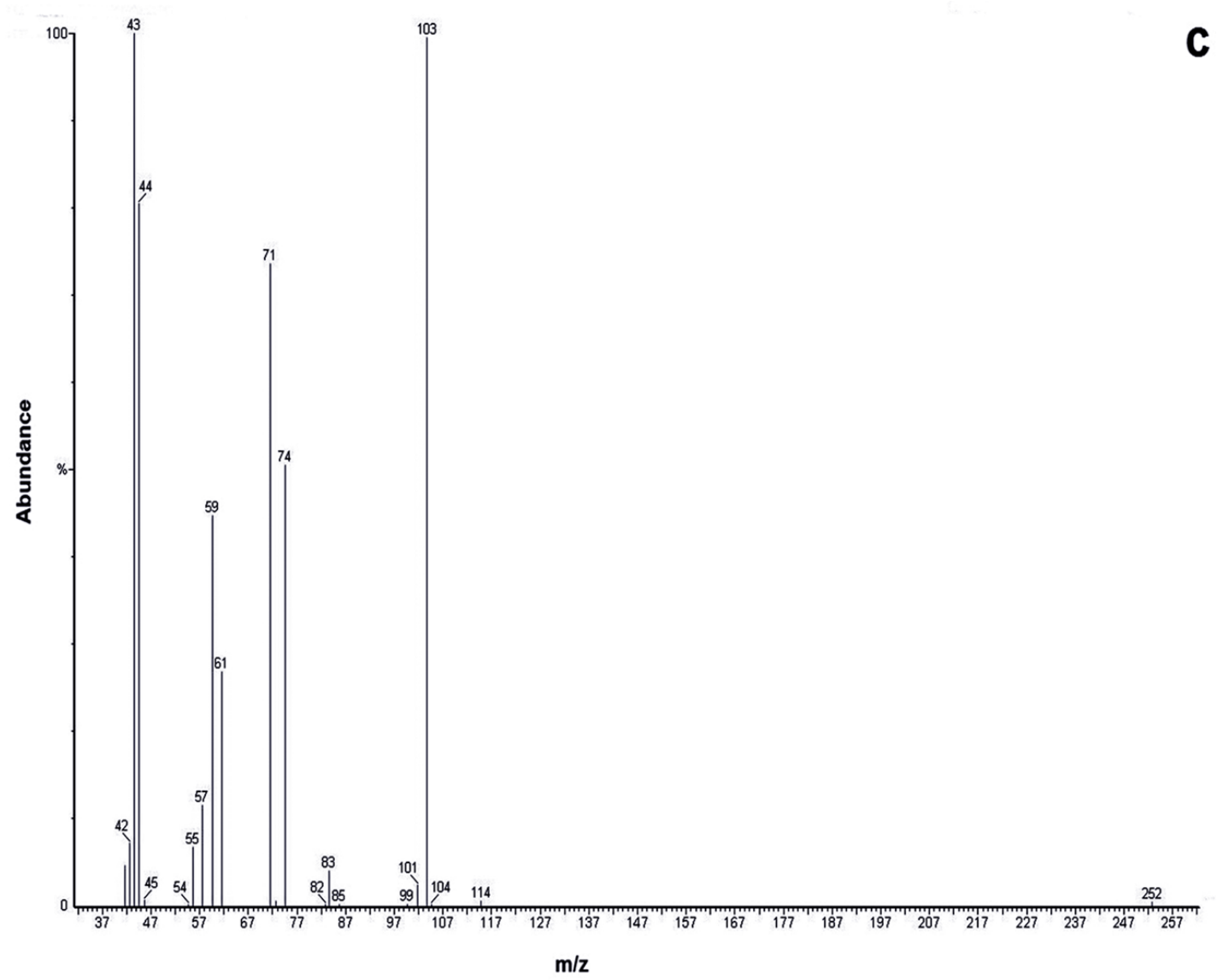
Sayed et al. (2009) have reported higher growth of *R. eutropha* ATCC 17697 and *A. latus* ATCC 29712 using organic nitrogen compared to inorganic nitrogen, while there was high PHB production by the strains in media supplemented with ammonium sulphate as nitrogen source. There are many reports to establish the effect of different nitrogen sources on *Bacillus* sp. (Borah et al., 2002, Yüksekdağ et al., 2004), *Streptomyces* sp. (Aysel et al., 2002), *Rhizobium* sp. (Mercan et al 2002), *A. latus*, and *R. eutropha* (El-Sayed et al., 2009), but none have reported the effect of different nitrogen sources on *Burkholderia* sp.

Results on optimisation of C:N ratio show that the optimal C:N ratio yields of the highest cell dry weight did not correspond to the highest PHA production. The cell dry weight of the strain decreased with increase in C:N ratio and the highest cell dry weight (8.15 g/l) was observed at C:N ratio of 13.24. However, the PHA accumulation in cell increased with increasing C:N ratio, till reaching the optimum values at C:N ratio of 39.72, and subsequently decreased up to C:N ratio of 79.44. At C:N ratio of 39.72, the PHA accumulation was 3.6 gL^{-1} of the medium corresponding to PHB content of 59 % (wt/wt) based on dry cell weight. Similar results in PHA study



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were reported by El-Sayed et al. (2009) with both *R. eutropha* ATCC 17697 and *A. latus* ATCC 29712 and found maximum PHB accumulation values at C: N ratio of 12.57. Based on the experiments in this study, the optimum pH, temperature, and incubation time for highest PHA accumulation were 7.0, 30°C, and 48 h respectively. The optimum inoculum concentration was found to be 5×10^4 cells in 100 ml medium for highest PHA accumulation. Lower inoculum concentration resulted in decreased PHA production due to delay in cell mass production. Further increase of inoculum size resulted in lower PHA accumulation. Similar results of influence of inoculum size on PHB production were also reported by Ramadas et al. (2010). An overall increase in PHA accumulation from 42 to 63% (wt/wt) was obtained under these optimised conditions.

GC-MS spectra of the PHA accumulated by various isolates as well as PHA standards confirmed that the polymer produced is mainly PHB except in case of VK-9 in which an additional peak at 5.27 min was recorded. The additional peak represents for P (3HB co HV) when compared with standard. Similar results were reported by Lakshman and Shamala (2003) for PHA produced by *R. meliloti* MTCC 100.

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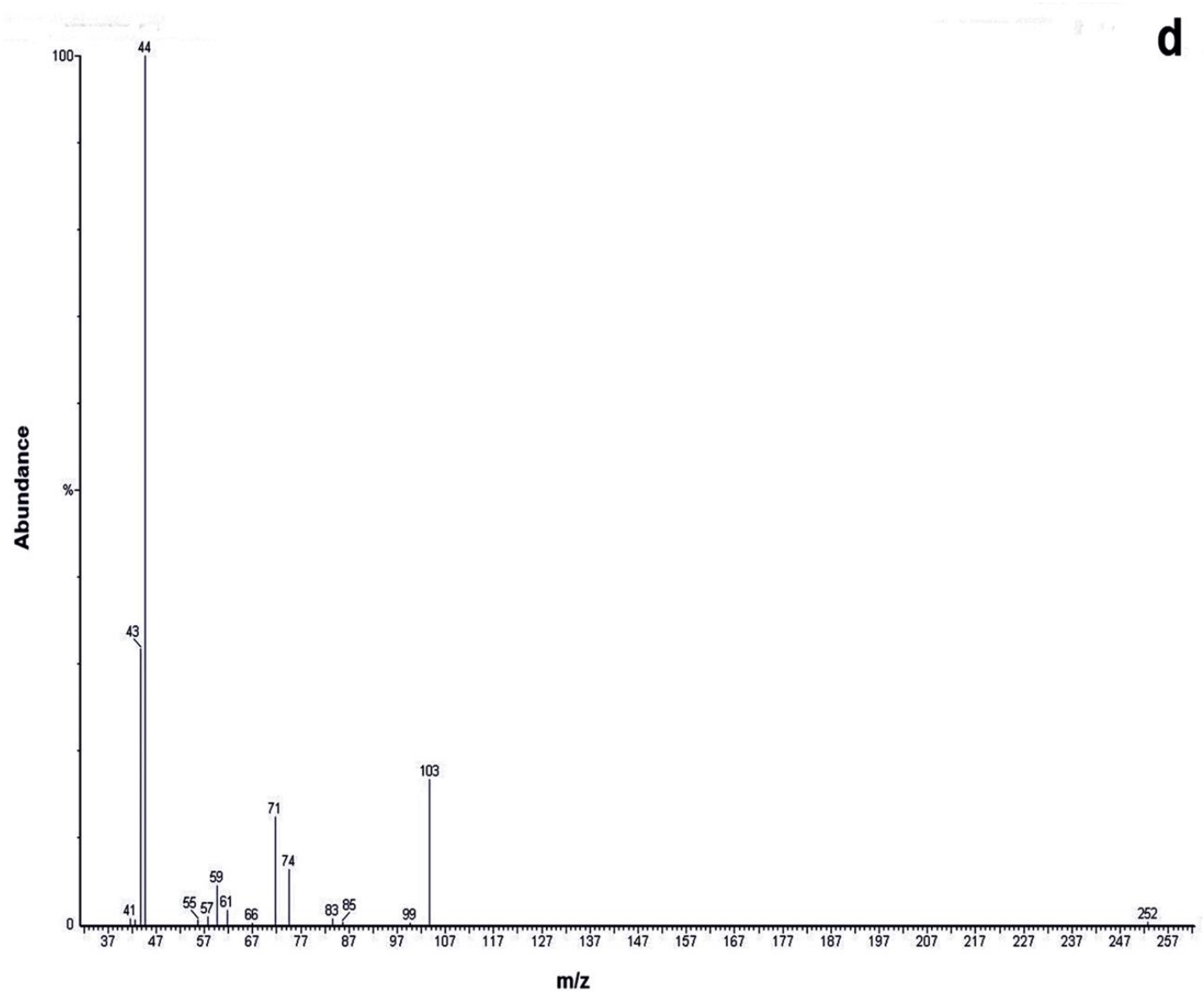

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