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Optimization of poly-β-hydroxybutyrate production by halotolerant bacterial strains isolated from saline environment

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Abstract: PHB is a biodegradable plastic which is becoming an environmentally friendly substitute to the synthetic plastics that are persistent and accumulate in large amounts and are non-degradable. PHB is a class of Polyhydroxyalkanoate which are similar to commercial plastics like polypropylene but with an added advantage of being biodegradable. To overcome the problem of commercializing PHB production by microorganisms because of the high cost involved, Halotolerant organisms can be used as they are easier to cultivate and do not require strict sterile conditions. In this present study PHB producing halotolerant bacterial strains were isolated from a marine environment and cultivated under saline conditions. The growth conditions of the bacterial strains were optimized for maximum production of PHB. The parameters such as pH, temperature, NaCl concentration, carbon sources, nitrogen sources and carbon and nitrogen ratio were optimized and studied. The growth conditions for each of the parameter were optimized and the PHB production was estimated for the bacterial strains under saline conditions. The optimum pH and temperature range yielded maximum PHB production of about 42 – 45 mg/100ml and 30 – 40 mg/100ml respectively. The perspective application of PHB could be in the medicinal field for manufacturing medical devices as implants for various surgeries such as dental, cranio – maxillofacial and dental surgeries.

Key words: Polyhydroxybutyrate; Halotolerant; Bacterial strains; PHB production; Bioplastics

Introduction

Today, plastic materials are part of humanities everyday life and are indispensible for numerous consumer goods and applications (Heinrich *et al.,* 2012). The diversity of the polymers and the versatility of their properties are used to make a vast array of products that bring medical and technology advances, energy savings and numerous other benefits (Devi *et al.,* 2014). An alternative must be developed to replace the use of synthetic plastic as well as to reduce the increasing environmental pollution (Bhagowati and Pabitra, 2013). A number of biodegradable thermoplastic polyesters are emerging out as a boon to overcome the problems of plastic waste accumulation. One of these promising materials is poly [(R)-3-hydroxybutyric acid] (PHB) (Ansari and Fatma, 2014). PHB is a thermoplastic, belonging to the family of polyhydroxyalkanoates (PHA). It is fully biodegradable polyester with optical activity, piezoelectricity, and very good barrier properties. PHB is a partially crystalline material with high melting temperature and high degree of crystallinity (Ghaffar, 2002).

Microorganisms provide a source of bioplastics and biopolymers (polysaccharides) from renewable sources. Although currently considerably more expensive than plastic derived from petrochemicals, bacteria have proved capable of yielding bioplastics with comparable properties. They have the additional advantage of being biodegradable. When grown under conditions where growth becomes limited through exhaustion of a key nutrient such as nitrogen or phosphorous and carbon substrate remains available, many prokaryotes can synthesize intracellular storage compounds. These act as carbon and energy reserves which can be utilized when balance growth is resumed (Sutherland, 2010). The major problem to the commercialization of PHB is its high production cost compared with conventional petroleum derived materials. In this context, extremophiles, especially halotolerant microorganisms, would be an important group to investigate (Ramezani *et al.,* 2014). There are several advantages to working with haloarchaeal strains rather than eubacteria. Due to the high salt concentrations they require in their growth medium to maintain cell wall stability, haloarchaeal strains do not require strict sterile conditions. Cell walls can also easily lyse in the absence of salt, especially in distilled water; this property enables the recovery of PHB or other PHA forms from extreme halophiles much more easily and economically than from eubacteria (Tekin *et al.,* 2011). Combining waste water with PHB production can be considered as a sustainable approach when dealing with environmental and economic issues (Yuan *et al.,* 2015).

In this present work, PHB producing halotolerant bacterial strains was enriched from saline soil

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environment. The growth parameters for PHB production by the bacterial strains were optimized under various pH, temperature, carbon, nitrogen and mixed carbon and nitrogen sources to yield higher production. The PHB products produced by the bacterial strains under saline conditions were confirmed by UV Spectrophotometry and FTIR Analysis.

Materials and Methods

Collection of soil samples

Soil samples were collected from the proximities of VGP Golden Beach and Neelankarai Beach, Chennai. The samples were collected in sterile zip lock covers and were immediately transferred to the laboratory and stored at 4°C.

Enrichment and isolation of PHB producing bacterial strains

1 g of soil sample collected was inoculated in Nitrogen-deficient medium without agar (Patnayak and Sree, 2005). The samples were then serially diluted in sterile distilled water and inoculated onto the Nitrogen Deficient Agar medium through pour plate technique. The plates were incubated at 37° C for 24 hours. Individual colonies were picked up from the bacterial consortium and plated on to the Nutrient Deficient Medium and incubated for 24 – 48 hours. The isolated bacterial strains were then identified by 16s rRNA sequencing and identified as NFKVG 8 – *Acinetobacter iwoffii* and NFKVG 10 – *Acinetobacter baumannii*.

Extraction of PHB

After 72 hours of incubation at 37°C in Nitrogen Deficient Medium, the culture broth was centrifuged at 8000 rpm for 15 minutes. The pellet along with 10 ml Sodium hypochlorite solution was incubated at 50°C for 1 hour for lyses of cells. The cell extract obtained was centrifuged at 12000 rpm for 30 minutes and then washed sequentially with distilled water, acetone and absolute ethanol. After washing, the pellet was dissolved in 10 ml chloroform and incubated at 50°C overnight and evaporated at room temperature. After evaporation, 10 ml of sulphuric acid was added to it and placed in water bath for 10 minutes at 100°C. This converts the PHB into crotonic acid, which gives maximum absorption at 235 nm using sulphuric acid as blank (Bhuwal *et al.,* 2013).

Quantification of PHB by Spectrophotometry

The extracted PHB was quantified by Crotonic acid assay (Hiremanth *et al.,* 2015). Crotonic acid powder was dissolved into 3 ml of sulphuric acid and standard solution of 0.1µg of Crotonic acid/µl of sulphuric acid was prepared. Working standards of 5, 10, 15, 20, 25, 30, 40, 50µg/3ml of sulphuric acid were prepared. Blank was prepared by adding 3 ml of sulphuric acid. The absorbance was measured at 235nm. Standard graph of concentration v/s absorbance was prepared.

Analysis of PHB production

FT – IR Spectroscopy

FT – IR analysis was carried out in an IR Affinity – 1, Shimadzu in Ethiraj College for Women, Chennai. The spectrophotometer was operated in the range of 4000 – 500 cm^{-1}.

Optimization of growth conditions

Different factors such as carbon and nitrogen source, C/N ratio, pH and incubation temperature play an important role in PHB production rate (Nehra *et al.,* 2012).

Effect of NaCl concentration

The effect of NaCl concentrations on PHB production was determined by growing the bacterial cultures of the PHB positive isolates in 100 ml of Minimal Salt Medium (MSM) (Kumar *et al.,* 2004). The PHB positive isolates were inoculated in MSM broth containing different concentrations of NaCl such as 3%, 5% and 7%. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 72 hours. After incubation, PHB produced by the isolates was quantified spectrophotometrically, and based on yield, the best salt concentration was determined.

Effect of different carbon sources

For carbon source optimization, individual cultures of the PHB producing bacterial strains were grown in MSM supplemented with different carbon sources such as glucose, lactose, maltose and sucrose at 2% concentration. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 72 hours. After incubation, PHB produced by the isolates was quantified spectrophotometrically, and based on yield, the best carbon source was determined.

Effect of different nitrogen sources

The PHB producing bacterial strains were inoculated in 100 ml of MSM broth containing the best carbon source and different nitrogen sources (ammonium acetate, ammonium nitrate, ammonium oxalate and yeast extract) at 1% concentration. After 72 hours of incubation at 30°C, PHB yield was determined for all the isolates and the best nitrogen source was selected on the basis of their yield.

Effect of carbon to nitrogen ration (C/N ratio)

As PHB accumulation has been found to be enhanced if the bacterial cells are cultivated in the presence of an excess carbon and limited nitrogen sources (Reddy *et al.,* 2009), therefore, in addition to the determination of the best C and N sources, the effect of different C:N ratios on PHB production was also determined. For this, the bacterial cultures were inoculated in MSM

supplemented with different ratios of concentrations of the best C and N source (C/N ratio as 10:1, 15:1, 20:1 and 25:1). Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 72 hours. After incubation, PHB yield was quantified spectrophotometrically and based on the yields the best C/N ratio was determined.

Effect of pH on PHB production

The effect of pH on PHB production was determined by growing the PHB producing bacterial cultures in 100 ml of MSM supplemented with the best C and N source having different pH, 6.0, 7.0 and 8.0. Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 hours. After incubation, PHB yield was quantified spectrophotometrically and the pH exhibiting maximum yield was determined.

Effect of temperature on PHB production

The effect of different incubation temperatures on PHB production was determined by inoculating the PHB producing bacterial cultures in MSM supplemented with the best C and N source and then incubating at different temperatures 25°C, 30°C, 35°C, 40°C and 45°C. After 72 hours of incubation at respective temperatures, PHB yield was quantified spectrophotometrically and based on the yields the optimum temperature for maximum PHB production was determined.

Results

Halotolerant Bacterial strains identified by 16s rRNA sequencing and identified as NFKVG 8 – *Acinetobacter iwoffii* and NFKVG 10 – *Acinetobacter baumannii* producing PHB were obtained through an extensive screening from saline soil. These organisms were maintained on nitrogen deficient agar slants at 4°C and maintained as glycerol stocks and revived monthly. The bacterial strains were isolated from the marine soil and the individual isolates were plated on to Nitrogen Deficient Medium with 3% NaCl for obtaining halotolerant strains which could yield higher amount of PHB. The halotolerant bacterial strains screened positive for PHB production and the growth was determined by Protein Estimation. The bacterial strains NFKVG 8 and NFKVG 10 were then optimized by varying different growth parameters for maximum production of PHB and their yield was estimated by UV Spectrophotometric analysis.

Growth pattern of PHB producing bacterial strains

The individual bacterial strains were grown in Minimal Salt Medium (MSM) to study the growth pattern and also to determine the PHB yield. It was shown that the individual bacterial strains showed maximum growth for the bacterial strain NFKVG 8 – 0.668 and NFKVG 10 – 0.621 respectively (Figure 1) and the PHB yield was seen to be maximum on the third day of incubation - 50 mg/100ml for the bacterial strain NFKVG 8 and 37 mg/100ml for NFKVG 10 (Table 1).

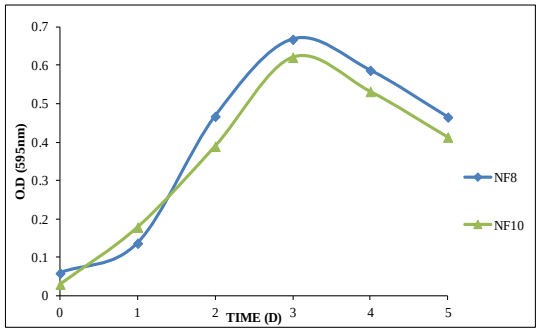

Figure 1: Growth pattern of PHB producing bacterial strains

Table 1: PHB yield by bacterial strains

Strain No	Growth (O.D at 540 nm)	PHB Yield (mg/100ml)
NFKVG 8	0.997	50
NFKVG 10	0.986	37

Polymer determination by FT IR Spectroscopy method

FT – IR spectra of the PHB production predicts the presence of functional groups of PHB (Sei *et al.,* 1994) i.e., aliphatic C – H, = O stretching, = C – H deformation and = C – O. PHB and their copolymers have known to contain these functional groups (Parshad *et al.,* 2001). PHB polymer extracted from the bacterial strains was used for recording the IR Spectra in the range of 4000 – 500 cm^{-1} (Figure 2). The spectrum showed intense absorption at the range of 1744 – 1030 cm^{-1} for the bacterial strains which shows the aliphatic nature of the PHB produced. The spectrum also showed the absorption bands of O – H stretching in the range of 3404 – 3411 cm^{-1} which was variable and sharp. In addition, the spectrum showed absorption bands of C – H stretching in the range of 2927 – 2860 cm^{-1} which was medium and variable (Table 2).

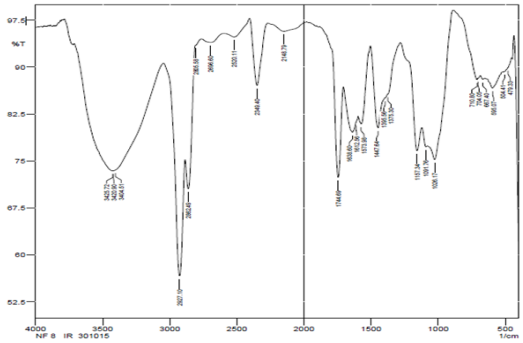

Figure 2: FT – IR spectrum of the bacterial strain showing PHB production

Table 2: Peak values of the bands and their corresponding functional groups for the presence of PHB production

Peak Value cm^{-1}	Functional Group	Intensity
3404.51	O – H stretch	Variable, sharp
2927.10	C – H stretch	Medium
2862.49	C – H stretch	Medium
1744.69	C = O aliphatic	Strong
1157.35	C – O esters	Strong

Quantification of PHB by UV – Spectrophotometry

To quantify the production of PHB, Crotonic acid was used as a standard at concentrations ranging from 5 – 50 µg/ml (Figure 3). Table 3 shows the optical density of the substrate increasing from 0.532 at 50µg/ml concentration to 0.971 at 500µg/ml of Crotonic Acid.

Table 3: Absorbance of Crotonic Acid at 235 nm

S.NO	Crotonic acid (µl)	Sulphuric acid (ml)	Concentration (µg/ml)	O.D value at (235 nm)
1	-	3	-	0
2	50	2.950	5	0.532
3	100	2.900	10	0.687
4	150	2.850	15	0.790
5	200	2.800	20	0.878
6	250	2.750	25	0.891
7	300	2.700	30	0.934
8	400	2.600	40	0.959
9	500	2.500	50	0.971

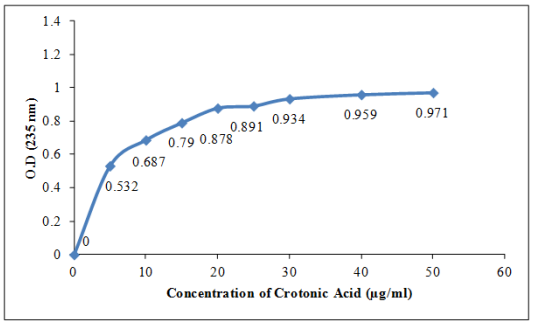

Figure 3: Standard graph of crotonic acid for the quantification of PHB

Optimization of growth conditions for the production of PHB

Effect of NaCl concentrations on PHB production

The bacterial strains were grown in the Mineral Salt Medium supplemented with 3%, 5% and 7% NaCl concentrations for 3 days (72 hours). It was seen that the maximum growth of the bacterial strains were seen in 3% NaCl concentration which also yielded maximum PHB production in the same concentration (Figure 4). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 58 mg/100 ml (Table 4).

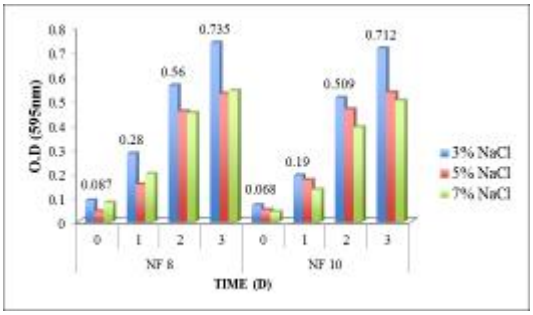

Figure 4: Total Protein content of the bacterial strains at different NaCl concentrations

Effect of different carbon sources

The bacterial strains were grown in the MSM in the presence of four carbon sources – glucose, lactose, maltose and sucrose at 2% concentration for 3 days (72 hours). It was seen that the maximum growth of both the bacterial strains were seen in Sucrose as the carbon source which yielded maximum PHB production (Figure 5). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 50 mg/100ml (Table.4).

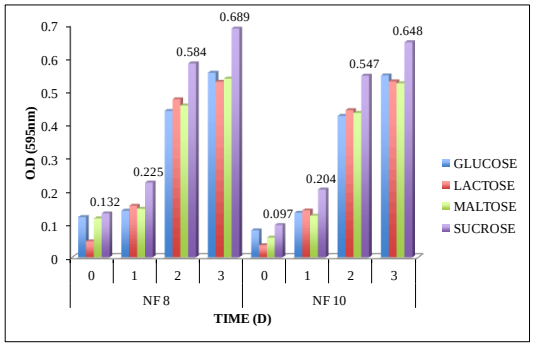

Figure 5: Total Protein content of the bacterial strains at different carbon sources

Effect of different nitrogen sources

The bacterial strains were grown in the MSM in the presence of four nitrogen sources – Ammonium acetate, Ammonium nitrate, Ammonium oxalate and Yeast extract at 1% concentration for 3 days (72 hours). It was seen that the maximum growth of the bacterial strains were seen in Yeast extract as the nitrogen source which yielded maximum PHB production (Figure 6). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 45 mg/100ml (Table.4).

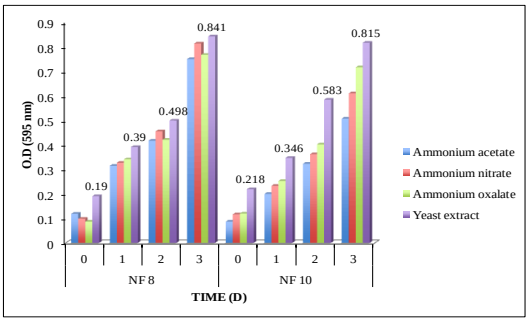

Figure 6: Total Protein content of the bacterial strains at different nitrogen sources

Effect of carbon: nitrogen ratio on PHB production

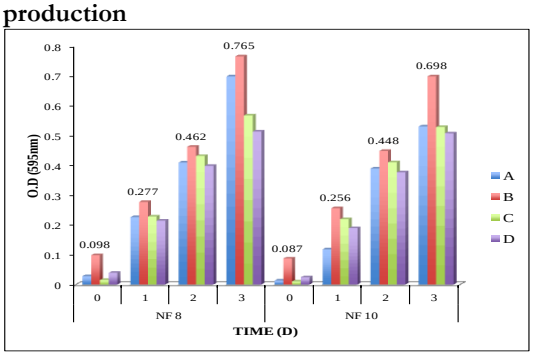

Figure 7: Total Protein content of the bacterial strains at different C:N ratio

The bacterial strains were grown in the MSM in four different ratios of C:N – A - 10:1, B - 15:1, C - 20:1 and D - 25:1 using the best carbon and the best nitrogen source for 3 days (72 hours). It was seen that the maximum growth of the bacterial strains were seen in 15:1 of C:N ratio which also yielded maximum PHB production (Figure 7). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 55 mg/100ml (Table.4).

Effect of pH on PHB production

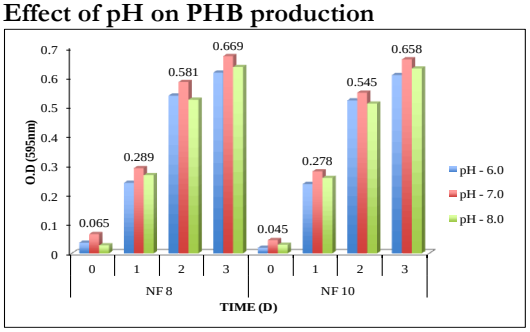

Figure 8: Total Protein content of the bacterial strains at different pH values

The bacterial strains were grown in the MSM in three different pH – 6.0, 7.0 and 8.0 using the best carbon and the best nitrogen source for 3 days (72 hours). It was seen that the maximum growth of the bacterial strains were seen in pH 7.0 which also yielded maximum PHB production in the same ratio (Figure 8). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 45 mg/100ml (Table 4).

Effect of temperature

The bacterial strains were grown in the MSM in five different temperature - 25°C, 30°C, 35°C, 40°C and 45°C using the best carbon and the best nitrogen source for 3 days (72 hours). It was seen that the maximum growth of the bacterial strains was seen in 35°C which also yielded maximum PHB production in the same temperature (Figure 9). Among the two bacterial isolates it was observed that NFKVG 8 showed the maximum yield of PHB production of about 40 mg/100ml (Table.4).

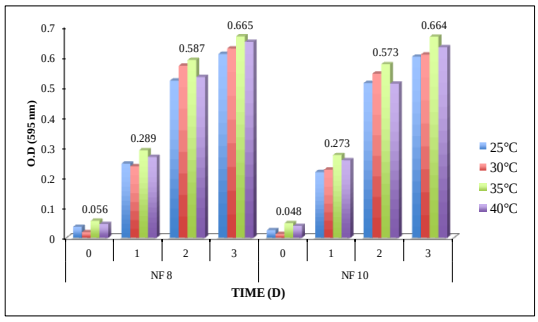

Figure 9: Total Protein content of the bacterial strains at different temperature ranges

Table 4: Quantification of PHB by the optimization of the various nutrient sources

Optimization Parameter	RANGES	PHB YIELD (mg/100 ml) NFKVG 8	NFKVG 10
NaCl concentrations	3%	58	54
	5%	49	46
	7%	23	18
Carbon sources	Glucose	20	17
	Lactose	19	18
	Maltose	12	9
	Sucrose	50	40
Nitrogen sources	Ammonium acetate	16	15
	Ammonium nitrate	21	19
	Ammonium oxalate	34	29
	Yeast extract	45	39
C:N ratio	10:1	38	36
	15:1	55	50
	20:1	32	30
	25:1	35	29
pH	6.0	35	34
	7.0	45	42
	8.0	30	27
Temperature	25° C	31	30
	30° C	29	25
	35° C	40	30
	40° C	20	19
	50° C	21	16

Discussion

A wide variety of bacteria are known to accumulate PHB granules intercellularly as an energy reserve material. Microbial species from over 90 genera have been reported to accumulate approximately 150 different hydroxyalkanoates as

polyesters granules (Steinbuchel, 1988). Rajendran *et al.,* (2013) reported the absorption band during FT – IR analysis from 2924 cm^{-1} to 2854 cm^{-1} stretching as aliphatic C – H band. The absorption band obtained at 1608 cm^{-1} as C = O stretching, the bands obtained at 1460 cm^{-1} as C – H of CH_3 stretching. In the present study, the absorption bands obtained in the range of 2924 cm^{-1} to 2860 cm^{-1} corresponded to the C – H aliphatic stretching and the absorptions bands for the C = O stretching were in the range of 1744 cm^{-1} to 1030 cm^{-1}. Shaaban *et al.,* (2012) used different carbon sources like glucose, fructose, sucrose, maltose and cellulose (1%) in their study in which Glucose showed the best PHB production of about 0.262 g/100ml culture. In the current work, sucrose was the best carbon source with PHB yield of 50 mg/100ml. They also reported that ammonium sulfate was the best nitrogen source that yielded maximum PHB production at 0.377 g/100ml. In the current work, it was seen that Yeast Extract was the best nitrogen source with PHB yield of about 45 mg/100ml. As PHB accumulation has been found to be enhanced if the bacterial cells are cultivated in the presence of an excess carbon and limited nitrogen sources (Reddy *et al.,* 2009), therefore, in addition to the determination of the best C and N sources, the effect of different C:N ratios on PHB production was also determined. Nehra *et al.,* (2012) used different C:N ratios (10:1, 15:1, 20:1 and 25:1) using the best carbon (glucose) and the best nitrogen source (ammonium sulfate) in the minimal salt medium. Amongst the different C/N ratios tested, 20:1 was found to be the best C:N ratio, supporting the highest PHB production (with an average of 79.29 mg/ml). In this study the various C:N ratios were 10:1, 15:1, 20:1 and 25:1 and it was shown that the optimum C:N ratio was 15:1 which also yielded maximum PHB production of 55 mg/100ml.

Lakshmi *et al.,* (2012) used different pH ranges like 5.0, 6.0, 7.0, 8.0 and 9.0 in their study for maximum production of PHB. It was reported that the optimum pH was 5.0 which yielded PHB of about 41g/l. In this study, the different pH ranges were 5.0, 6.0 and 7.0 and the optimum pH was 7.0 with PHB production of about 45 mg/100ml. Bhuwal *et al.,* (2014) optimized different incubation temperatures for the production of PHB. The effects of different temperatures ranging from 25°C to 55°C were studied for PHB production. It was observed that optimum temperature was at 35°C which yielded PHB of about 4.274g/l.

In the present study different incubation temperatures - 25°C, 30°C, 35°C, 40°C and 50°C were used for the optimum PHB production. The maximum production of PHB was observed at 35°C which showed PHB production of 40 mg/100ml. Prasad and Sethi (2013) reported that the PHB positive isolates were identified to be *Pseudomonas sp.* based on the Bergey's manual of determinative Bacteriology. In the present study, the bacterial strains NFKVG 8 and NFKVG 10 were biochemically characterized and molecularly identified as *Acinetobacter iwoffii* and *Acinetobacter baumannii.*

Conclusion

PHB is the most popular and the best characterized polymer belonging to the class of polyhydroxyalkanoates. It could be used for applications similar to those of common plastics and could fit well into new waste-manageable strategies as well. The recovery of PHB from the cell wall of extreme halophiles is much easier when compared with eubacteria. Hence in this study, halotolerant bacterial strains were isolated from marine soil and the growth parameters were optimized for the maximum production of PHB. The future perspectives of this study would be to employ novel methods of PHB production which are both cost-effective and environmentally friendly.

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