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Production of Polyhydroxybutyrate (biopolymer) by *Bacillus tequilensis* NCS-3 Isolated from Municipal Waste Areas of Silchar, Assam

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Abstract: ***Polyhydroxybutyrate (PHB) is a biodegradable polymer produced by many bacteria. It can be used as an alternative to petroleum-based commercial plastics as they can be easily degraded by the soil microorganisms. The present work focuses on screening a potential bacterium for PHB production. In this study, 50 bacterial colonies from the soil samples were isolated and screened for PHB production. They were grown in basally-defined M9 medium (DM9 medium) and screened for biosynthesis of PHB using Sudan Black B stain. 20 were found to be able to produce PHB and among them, isolate 3 showed highest productions of 1.0g/l PHB from 1.92g/l DCW (dry cell weight). Isolate 3 was characterized as Bacillus tequilensis NCS-3 based on 16S rRNA gene sequence. This isolate was selected to optimize its culture conditions using different carbon sources, nitrogen sources, pH and temperatures. After optimization, the isolate yielded maximum amount of 1.75g/l PHB from 2.01g/l DCW at pH 6 and incubation temperature of 30^{0}C using fructose and tryptone as carbon and nitrogen sources. PHB production increases from 52% to 87% after optimization, which indicates that Bacillus tequilensis NCS-3 was a potent PHB producer.***

Keywords: Polyhydroxybutyrate (PHB), Biopolymer, *Bacillus tequilensis* NCS-3, 16S rRNA, DM9 medium

1. Introduction

Accumulation of conventional plastics, which are not degradable by microorganisms, are increasing day by day despite their wide range of utilities in the civilization of mankind. An alternative approach is to replace it with biopolymer (natural polymer) which can be degraded easily by microbes leaving non-toxic wastes, and completely recyclable into organic waste.

Polyhydroxyalkanoates (PHAs) is a class of natural polyesters (biopolymer) that many microorganisms in the environment accumulate in the form of intracellular granules to store carbon and reducing equivalents [1]. PHAs are non-toxic, biocompatible and biodegradable thermoplastics that can be reproduced from renewable resources [2].They can be easily degraded in soil and sewage, and can be processed using the extrusion technology that is currently being used in making polyethylene or polypropylene films [3].

Polyhydroxybutyrate (PHB) is the best known polyhydroxyalkanoate (PHA) which is now well recognized that this lipid inclusion is accumulated by many bacteria as they enter the stationary phase of growth to be used later as an internal reserve of carbon and energy [4]. Some of the microorganisms that produce PHB as energy reserves includes *Alcaligenes, Azotobacter, Bacillus, Nocardia, Pseudomonas, Rhizobium,* etc. [5]. It is generally accepted that microorganisms isolated from a natural environment poor in nutrient sources (from soil or spring water) exhibit higher survival ability than those living in the alimentary tract of higher organisms [6]. In the presence of an abundant source of carbon, some bacteria can accumulate upto 60-80 % of their weight as PHB under nutrient limited conditions [7]. The PHA content and its composition are influenced mainly by the strain of the microorganism, the type of substrate employed and its concentration, and other growth conditions [8].

Although PHAs can replace the commercial petroleum-based plastics, its widespread use is still hindered because, the use of sterile equipment, defined substrates and downstream processing led to high costs of PHA production, which limits the industrial application of PHA [9]. In order to reduce the production cost, a lot of efforts are being carried out so that PHB can be more effectively used in place of petroleum-based plastics. Some of the strategies scientists have been exploring involves utilizing low cost substrates (e.g. industrial effluents, waste water medium, agricultural waste materials, etc.), mixed microbial cultures, optimization by genetic engineering, optimization of the culture conditions (like pH, temp., C-sources, N_2-sources, etc.), etc.

Soil and activated sludge serves an important bioresource as majority of PHB producing bacteria was isolated from these environments [10]. The hypothesis of this study was that these environments should provide conditions under which diverse PHB producing bacteria are enriched. So, the aim of the present study was to screen and isolate efficient PHB accumulating bacteria from the soil samples of municipal waste areas of Silchar, Assam and to evaluate the appropriate culture conditions of the bacteria for efficient PHB production.

2. Materials and Methods

2.1 Isolation of PHA producing microorganism

Soil samples from municipal waste areas of Silchar, Assam were taken 0-10cm beneath the surface of the soil. Samples were stored at room temperature until analysis. 1.0g of sample was serially diluted in sterile distilled water and

plated onto nutrient agar medium and incubated at 37°C for 48 h. Morphologically dissimilar colonies were individually picked and sub cultured 4-5 times on nutrient agar plates. The pure cultures isolated were maintained in Luria Bertani (LB) agar slants and preserved in 50% glycerol stock at -80°C. PHA producing strains were screened using Sudan Black B stain and an efficient PHA producer was selected based on the amount of PHA produced.

2.2 Characterization of the isolated bacteria

The morphological and physiological properties of the bacterium were investigated according to Bergy's manual of determinative bacteriology [11].

2.3 16S rRNA gene sequencing

The genomic DNA was extracted from 1 ml broth culture of the isolate using HiPurA™ Bacterial genomic DNA Extraction Kit. From the genomic DNA, nearly full-length 16S rRNA sequences were amplified by PCR using primers 27F and 1492R [12]. PCR was performed with a total volume of 50 µl containing 1 µl of 30 ng DNA, 2.5 µl of 2.5 mM dNTP, 1 µl of 100 pmol of each primer, 5 µl of 10 u buffer, 0.5 µl of 1.5 U Taq polymerase and 40 µl nuclease free water. Amplifications were carried out with the following temperature profile: 5 min at 95°C, 30 cycles of denaturation (60 s at 94°C), annealing (60 s at 55°C), extension (2 min at 72°C) and a final extension for 7 min at 72°C. Amplified products were separated on 1% agarose gels in 1x TAE buffer at10 V cm^{-1} for 90 min and observed with a UV transilluminator and documented with GelDocXR software (Biorad). The amplification product was purified using Genejet Gel Extraction PCR purification kit according to the manufacturer's instruction. The purified PCR product was sequenced by ABI 3500 Genetic Analyser. The 16S rRNA gene sequence analysis was carried out using NCBI-BLAST (National Centre for Biotechnology Information http://www.ncbi.nml.nih.gov) program. The sequences were aligned and the phylogenetic tree was constructed using the neighbor-joining method using MEGA 4.1: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1

2.4 Growth medium and culture conditions

Cultivation was carried out in LB broth for 24 h at 37°C on a rotary shaker at 150 rpm/min. Primary inoculum development was carried out in growth medium containing (g/l): beef extract, 1; peptone, 5; yeast extract, 2; NaCl, 5; with a pH of 7.0. DM9 medium (basally- defined M9 medium) was used for cell growth and PHB production [13].The composition of the medium was (g/l): glucose, 4; NH_4Cl, 1; Na_2HPO_4, 7; NaH_2PO_4, 3; 10ml 0.01M $CaCl_2$ and 10ml 0.1M $MgS0_4.7H_20$. The cultures from the primary cultivation were aseptically centrifuged at 2000 × g for 30 min to separate the biomass. The biomass was then inoculated into the production medium and grown for 48 h at 30°C on a rotary shaker at 150 rpm.

2.5 Extraction of PHA from the isolate

The culture from the production medium after 48 h of growth was screened for PHA production by staining with Sudan black B. Extraction of PHA from *Bacillus tequilensis* NCS-3 was carried out using the Hypochlorite method with a slight modification [14]. For this, the isolate was grown in 250 ml Erlenmeyer flasks containing 50 ml of the DM9 medium and incubated at 30°C for 48 h on scientific environmental shaker at 160rpm. 10ml of the cell suspension was centrifuged at 6000rpm for 10mins. The cell pellet was washed once with 10ml saline and was recentrifuged to get the pellet. Cell pellet was then suspended in 5ml sodium hypochlorite (4% active chlorine) and incubated at 37°C for 10 min with stirring. This extract was centrifuged at 8000rpm for 20mins and the pellet of PHA was washed with 10ml cold diethyl ether. The pellet was again centrifuged at 8000 rpm to get the purified PHA and was dried to constant weight at 60°C.

2.6 Analytical methods

For the determination of the dry cell weight (DCW), the fermentation broth culture was used. By measuring the dry cell weight (DCW), the cell concentration was determined, for this, 5ml culture broth was centrifuged, washed with distilled water and dried at 60°C until the weight does not decrease further. The residual mass was defined as total DCW minus PHB weight;PHB (%) was defined as the percentage of the ratio of PHB to DCW.

2.7 Optimization of culture conditions

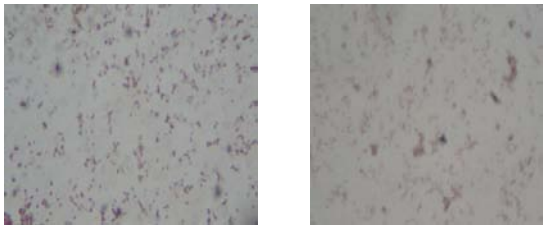
5ml of the production medium was inoculated with the bacterial culture and incubated at 37°C for 48 h in shaker under different conditions of growth. To optimize the culture conditions, different carbon sources, nitrogen sources, pH and temperatures were tested for PHB production. Among the carbon sources, glucose, mannitol, fructose, sucrose and starch were tested at a fixed concentration of 4g/l; peptone, yeast extract, tryptone, urea and ammonium chloride were among the nitrogen sources tested at fixed concentration of 1 g/l; pH gradients evaluated were 5, 6, 7, 8, 9 and temperatures selected were 20°C, 25°C, 30°C, 35°C and 40°C.

3. Results

3.1 Selection of *Bacillus tequilensis* NCS-3 as a potent PHB producer

50 bacterial colonies were isolated from the soil samples collected from municipal wastes areas of Silchar region of Southern Assam. These were screened to select the potent PHB producing isolate. PHB production was verified by staining with Sudan Black B stain. Stained PHB granules appeared as blue black droplets inside the pink coloured cells after 48 h of growth in the production medium (Fig. 1 a, b). According to the results of Sudan Black B staining, 20 among the 50 isolates were found to be PHB positive. These 20 isolates were again grown in the DM9 medium for estimating the amount of PHB produced and among them 5 isolates viz. 3, 4_1, sh-5, S_9 and S_{13} were found to produce highest amount of PHB. Out of this, isolate 3 showed highest

productions of 1.0g/l PHB from 1.92g/l DCW (52% of the DCW). According to the above mentioned results, isolate 3 was chosen as a potent bacterium for producing PHB and hence this was selected to optimize its culture conditions.



(a) (b)

Figure 1: Sudan Black B results (a) PHB positive (b) PHB negative

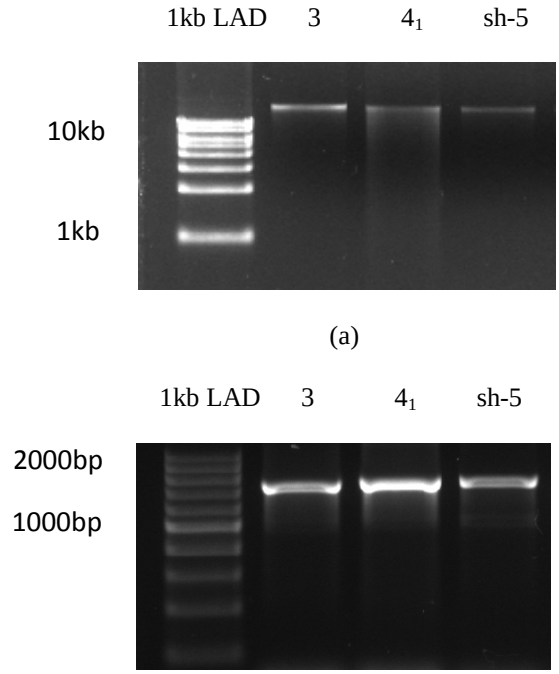


Figure 2: (a) Genomic DNA bands of the isolates 3, 4_1 and sh-5 (b) Amplified PCR product of 16S rDNA gene of the isolates 3, 4_1 and sh-5

3.2 Identification and characterization of the isolate *Bacillus tequilensis* NCS-3

The isolate 3 was identified using a series of biochemical tests and morphological characteristics (Table 1). Microbiological properties were investigated according to the methods described in ‘‘Bergey’s manual of determinative bacteriology” [11] and the organism was identified as a member of the genus *Bacillus* and confirmed the species *tequilensis*. Further characterization was confirmed with 16S rDNA sequence from the isolated DNA bands (Fig. 2 a, b). The almost complete 16S rDNA gene sequence of the strain NCS-3 following pair-wise alignment exhibited 99% similarity with the partial sequence of *Bacillus tequilensis* strain 10b, 99% similarity with *Bacillus subtilis* strain JCM 1465, 99% similarity with *Bacillus subtilis* strain NBRC 13719, 99% similarity with *Bacillus subtilis* strain OS-6.2, 99% similarity with *Bacillus subtilis* strain DSM 10, 99% similarity with *Bacillus subtilis* strain OS-44.a, 99% similarity with *Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28, 99% similarity with *Bacillus subtilis* strain BCRC 10255, 99% similarity with *Bacillus subtilis* strain IAM 12118, 99% similarity with *Bacillus subtilis* strain OS-109. The complete 16S rDNA gene sequence was submitted to the NCBI database and the sequence was assigned the accession no. KM117225 (Fig. 3). The phylogeny based on 16S rDNA sequence of *Bacillus tequilensis* NCS-3 and related taxa was shown in Fig. 4.

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TCGGGCGGGCCTAATAATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCC
TGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAG
ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAAC
CGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGAC
CCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGAACTCTCCTACGGGAGGCAGCAAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT
AAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGC
TCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAG
CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTGCGCAAGACTGAAACTCAAAGGAATTGACGA
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGGAGATAGGTAC
GTCCCCTTCGGGCGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGGGATGACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGA
GGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAA
CTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG
TTTGTAACACCCGAAGTCGGTGAGGTAACCTTTAGGAGCCAGCCGCCGAA
GTGTCCA

Figure 3:16S rDNA sequence of *Bacillus tequilensis* NCS-3

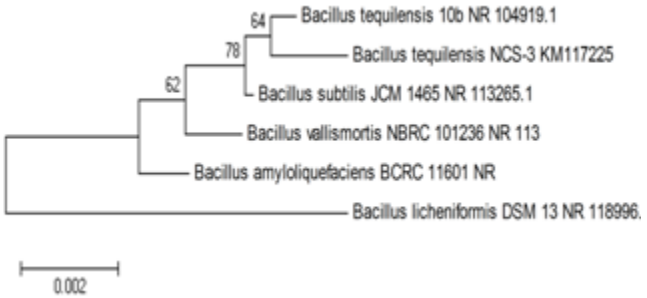

Figure 4: Phylogenetic analysis of 16S rDNA sequence of *Bacillus tequilensis* NCS-3 and related taxa. The tree was generated by neighbor-joining method using MEGA 4.1: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1

Table 1: Phenotypic characterization of *Bacillus tequilensis* NCS-3

Traits	*Results*
Colony morphology	
Colour	Yellowish
Shape	Circular
Elevation	Raised
Margin	Entire
Transparency	Opaque
Surface	Smooth
Gram's test	+
Motility	+
Anaerobic growth	-
Growth at ^0C	
30	+
35	+
40	+
Growth at pH	
6	+
7	+
8	+
Catalase	+
Oxidase	+
Starch hydrolysis	+
Citrate Utilization	+
Nitrate reduction	+

+ = positive, - = negative

3.3 Effect of different carbon sources, nitrogen sources, pH and temperature on PHB accumulation

In this investigation, the culture was grown for 48 h at 30^0C and the effect of carbon sources (glucose, mannitol, fructose, sucrose and starch) at a fixed concentration of 4g/l, on the production of PHB by isolate 3 were evaluated. Of the various carbon sources, fructose positively affected PHB production with a concentration of 5.55g/l when it was the sole carbon source. Moreover, the isolate 3 was also able to take up some of the other carbon sources (like starch and sucrose) as nutrients for producing PHB. However, low PHB production and low cell growth were observed when the carbon source was glucose and mannitol (Table 2). Therefore, isolate 3 adapted to use fructose as carbon source resulting in greater PHB production than using other carbon sources.

PHB production was also significantly affected when different nitrogen sources were added to the production medium as also shown in Table 2. The nitrogen sources were taken at a fixed concentration of 1 g/l and the isolate was grown for 48 h at 30^0C in the DM9 medium. According to the results obtained, almost all the nitrogen sources except urea enhanced PHB production and tryptone had the largest effect producing 7.65 g/l PHB concentration from 10.02 g/l DCW. However, urea did not show any PHB production, this may indicate that isolate 3 can't take up urea as nitrogen source for PHB production.

The influence of pH on PHB production by the isolate 3 was studied and highest production of 5.75 g/l PHB and 4.81 g/l PHB was obtained at pH 6 and 7 respectively. The result of PHB yields at different temperature conditions (20^0C, 25^0C, 30^0C, 35^0C and 40^0C) was also examined. Table 2 shows that the optimal temperature was 30^0C producing 5.72g/l PHB from 8.21g/l DCW.

Table 2: PHB production at different carbon sources, nitrogen sources, pH and temperatures

Parameters		*DCW (g/l)*	*PHB concentration (g/l)*	*PHB content (%)*
Carbon sources	Glucose	1.06	0.35	33.0
	Fructose	12.23	5.55	45.3
	Mannitol	1.01	0.42	41.5
	Sucrose	8.21	2.45	29.8
	Starch	9.03	3.20	35.4
Nitrogen sources	Peptone	9.20	4.51	49.0
	Yeast extract	7.01	3.92	55.9
	Tryptone	10.02	7.65	76.3
	Urea	5.12	*ND	*ND
	Ammonium chloride	7.81	5.25	67.2
pH	5	9.20	4.01	43.5
	6	7.65	5.75	75.1
	7	7.25	4.81	66.3
	8	5.03	2.55	50.6
	9	9.71	4.65	47.8
Temperatures	20^0C	10.11	5.45	53.9
	25^0C	7.65	3.87	50.5
	30^0C	8.21	5.72	69.6
	35^0C	6.09	2.34	38.4
	40^0C	8.15	2.01	24.6

*ND=Not Detected

4. Discussion and Conclusion

Concern over petrochemical plastics in the environment, has created a renewed interest in biologically derived polymers. PHAs can serve as an efficient alternative to petroleum based non-degradable polymers [15]. Many bacterial strains which have potential to produce PHA have been isolated and identified from different origins but, still screening of a novel bacterium remains untapped [16]. The sheer diversity of the microbial community calls for the identification of bacteria capable of producing large amounts of PHB utilizing cheap nutrient sources. This requires careful optimization and analyses of conditions under which PHB synthesis is maximized.

In this study, a new potential PHB producer was isolated from the municipal waste areas of Silchar, Assam and its culture conditions were optimized for efficient PHB production. On the basis of morphological information, cultural studies, biochemical data and molecular analysis, the new bacterium isolated, clearly belonged to the genus *Bacillus*. Phylogenetic analyses of 16S rRNA demonstrated that this bacterium grouped with *B. tequilensis* shows a well-defined taxon that deserves the rank of species. The isolate was termed as *B. tequilensis* NCS-3 and found to be aerobic, gram positive, motile, rod shaped, spore forming, oxidase positive, catalase positive and positive for citrate utilization test. This bacterium can also reduce nitrate to nitrite and can hydrolyse starch too. Production of PHB has also been reported in different *Bacillus* species i.e, *B. megaterium* [17], *B. subtilis* [18], *Bacillus* species [19, 20], *B. mycoides* [21], etc.

PHB production is largely dependent on the type of the carbon source utilized by the bacteria. The course of PHB concentration and PHB formation rate was shown in Table 2. The maximum PHB production was attained (45 % DCW) when fructose was used as a sole carbon source. The PHB production was clearly decreased when fructose was replaced by glucose, sucrose, mannitol and starch that were 33, 29, 41 and 35 % DCW, respectively. Similar results were observed in *Bacillus megaterium* and other *Bacillus* sp. [22]. Reports were also there which showed fructose as an effective nutrient for producing PHB [23]. Using sugars as carbon source for PHB production can also be useful for saving energy required for liquefaction and saccharification.

Supplementation of different nitrogen sources in DM9 medium clearly shows the influence of nitrogen in the production of PHB. The cell biomass varied from 5.12g/l to 10.02g/l and maximum biomass was obtained with tryptone as shown in Table 2. Previous reports suggested that complex nitrogen sources increased the yield of PHB [19] whereas better yield of PHB was obtained by *Bacillus*, *Staphylococcus* and *Pseudomonas* using ammonium sulphate and ammonium phosphate as nitrogen sources than that of yeast extract [21]. Peptone is also a good source of nitrogen which favours the growth and PHB production by *Azotobacter chroococcum* [24]. In the present study, *Bacillus tequilensis* strain showed substantially higher biomass (10.02g/l) in turn, high PHB accumulation (67.3%) when compared with other *Bacillus sp* strains like *Bacillus sp* INT005 (35.30%) [25], *Bacillus cereus* SPV (41.90%) [8], *Bacillus cereus* CFR06 (46.0%) [26] reported so far.

Temperature and pH also plays an important role in the production of PHB and therefore its influence was studied in optimizing the culture conditions of the bacteria. PHB production by *Bacillus tequilensis* NCS-3 was examined at 20^0C, 25^0C, 30^0C, 35^0C and 40^0C. The optimal temperature found was 30^0C producing 5.72g/l PHB from 8.21g/l DCW. Similar results were obtained by other *Bacillus sp.* which showed that 30^0C was a favourable temperature for PHB production [23, 27]. The influence of pH on PHB production by *Bacillus tequilensis* NCS-3 strain was also optimized and highest PHB production of 5.75g/l and 4.81g/l was obtained at pH 6 and 7. It has been reported that pH value ranging from 6.0-7.5 is optimum for PHB production by *Alcaligenes latus* [27]. PHB production occurs at pH 6.4 and that the lack of polymer accumulation at higher pH value may be due to an effect on the degenerative enzymes of polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis [28].

Based on the above results, it could be concluded that optimum culture conditions for effective PHB production by *Bacillus tequilensis* NCS-3 was pH 6 at 30^0C by utilizing fructose and tryptone as carbon and nitrogen sources respectively. Before optimizing the culture conditions, it produces 1.0g/l PHB from 1.92g/l DCW but after optimization, it produces 1.75g/l PHB from 2.01g/l DCW i.e PHB production increases from 52% to 87% of DCW. Therefore, selection of efficient PHB producing bacteria and on optimizing the most favourable conditions for PHB production is very important for successful production of biodegradable plastics.

5. Future Scope

This study suggests that *Bacillus tequilensis* NCS-3 is a potential PHB producer that could be utilized for industrial production of bioplastic and by utilizing its optimized culture conditions; we could somehow reduce the high cost of PHB production.

6. Acknowledgement

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