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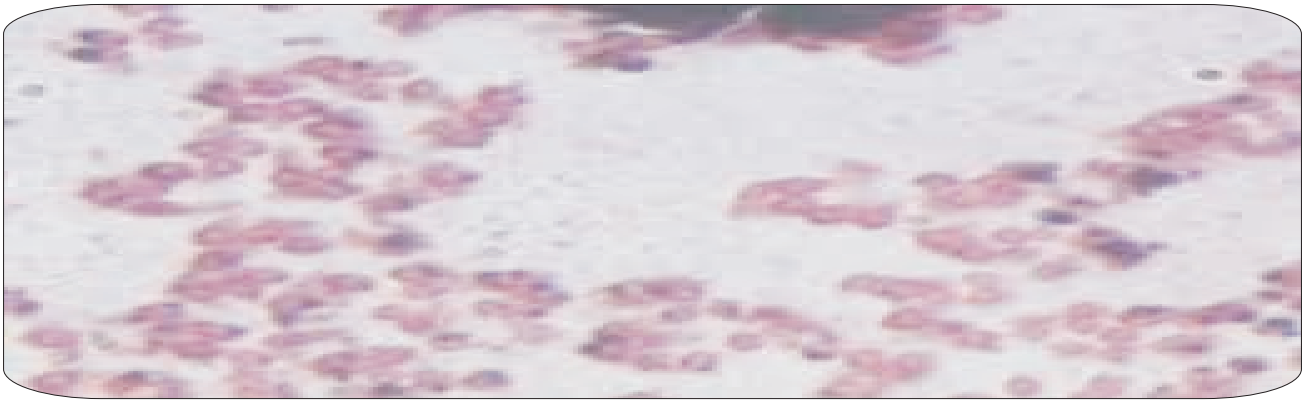

SCREENING AND OPTIMIZATION OF POLYHYDROXYBUTYRATE (PHB)FROM AZOTOBACTER SPP

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ABSTRACT

Azotobacter spp is a obligate aerobe that is able to fix nitrogen and has a remarkable machinery to synthesis an intra cellular polyester Poly B hydroxybutryate.This experiment was done to qualitatively and quantitatively screen the soil isolates for PHB production. Study on the growth and production of PHB was done at standard pH and temperature .Optimization of the media with various physico chemical parameters was done for increased production of PHB. Production of PHB serves as an advantage to the bacteria as it would help the bacteria to tolerate abiotic environmental stress and perform as a potent bioinoculant. The bacteria can also be biotechnologically explored for bioplastic production and put an end to plastic pollution.

KEYWORDS :Azotobacter , PHB, Bioplastics.

1. INTRODUCTION

Azotobacter is an obligate aerobic bacterium that able to fix atmospheric nitrogen and grow under microaerophilic conditions. This microorganism is able to synthesize three molecules of

important biotechnological and biochemical applications; the extracellular polysaccharide alginate, siderophores compounds and polyhydroxybutyrate(PHB). The latter is biodegradable thermoplastic polyester analogous or better than those of chemically synthesized and petroleum based polymers such as polyethylene and polypropylene[1][2]. PHB is a biopolymer that has been implicated in supporting nitrogen fixation[3], biodegradable thermoplastic material for management including plastics, films, and fiber strategies, and biocompatibility in the medical devices [4][5][6]. This polymer is accumulated due to depletion of nitrogen, phosphorous or oxygen to form carbon and energy reserve material.

PHB is considered to be an ideal storage material because of its highly reduced and water insoluble character. Therefore, no osmotic pressure effects are induced inside the cell. This polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage material accumulated intracellularly to be metabolized when other common energy sources are not available [7][8]. Conditions for optimal production of PHB usually include an excess of carbon source and exhaustion of a single nutrient such as nitrogen, sulphur, phosphate, iron, magnesium, potassium or oxygen [9]. Apart from advantages mentioned above, several reasons can be proposed to explain for selecting PHB producing Azotobacter spp., because of its ubiquitous presence in soil, resistance to heat and ability to germinate and grow in unfavorable conditions.

PHB has also been found in numerous heterotrophic and autotrophic aerobic bacteria[10], gliding bacteria [11], Cyanobacteria [12] and many other prokaryotes. The main advantage of this polymer (Poly β-hydroxybutyrate) is that, since they are of biological origin, they degrade naturally and completely to $C0_2$ and H_20 under natural environment by enzymatic activities of microbes.

MATERIALS AND METHODS

Isolation & Enumeration of *Azotobacter* spp., from soil samples

Azotobacter was enumerated from each sample by serial dilution and spread plate method. For this, 1.0 gm of soil sample was mixed with 9 ml of sterile distilled water and was thoroughly shaken in a rotary shaker for 30 minutes at room temperature. From this soil suspension, 1.0 ml was serially diluted with sterile distilled water to obtain 10^{-2} - 10^{-8} in 2-fold dilution format. From each of these dilutions, 0. 1 ml was spread on Jensen's medium and incubated at 27-30˚C for 48-72 hours.

Morphological and Biochemical characterization

Bacterial colony morphology of the soil isolates on Jensen's isolation agar was examined for its colony form, shape, size, colour, elevation, and texture . Individual *Azotobacter* spp. soil isolates were sub cultured and stored adopting standard techniques . From subcultures, an individual aliquot was tested for Gram's staining reaction and motility adopting the methodology described in Bergey's manual of Determinative Bacteriology [13]. *Azotobacter* reference strain MTCC 124, purchased from Institute of Microbial Technology (IMTECH), Chandigarh, India was included as a positive reference control in all tests.

Individual soil isolates were subjected to a variety of biochemical analysis to confirm the genus (Azotobacter) of the isolates [14]. Biochemical & physiological tests that were used to characterize the soil isolates includes, Indole, Citrate utilization, Nitrate reduction, Carbohydrate fermentation, Catalase and Oxidase tests.

Qualitative Screening for the Production of PHB using Sudan Black Staining Technique [15]:

The isolated bacterial strains were screened for PHB production. As a preliminary step, screening of PHB producers was carried out using viable colony staining technique. The cultures were grown on Minimal Salt Medium (MSM) described by Gracia and colleagues, (2002) supplemented with glucose (2%) as a sole carbon source, incubated at 30^{0}C for 48hrs. After incubation, the plates were flooded with Sudan black B solution for the detection of microbial intracellular lipid granules and kept undisturbed for 20 minutes. The excess of Sudan black solution was drained off. Viable colony staining technique was selected in order to reveal the different pattern of Sudan black absorption seen on the agar plates such as Maximum, Moderate and Minimum absorption.

Extraction of Poly- -hydroxybutyrate

PHB produced from the selected and standard isolates were extracted by the following procedure. The isolates were subjected to quantification of PHB production [16][17]. The bacterial cells containing the polymer were centrifuged at 10,000 rpm for10 min and the pellet was re suspended into alkaline sodium hypochlorite (pH 10.0-10.5 NaOCl content 5.25%-5.5%) and incubated at room temperature for 1 hr. The whole mixture was again centrifuged at 10,000 rpm for 10min and the supernatant was discarded. The cell pellet containing PHB was again washed with water, alcohol and acetone. Finally, the polymer was dried for 2 hours at 105oC and then weighed. Dry weight of extracted PHB was estimated as g/L. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHB [18].

Estimation of Dry Cell Weight (DCW):

After 48 hrs incubation at 37° C, culture medium was collected and centrifuged at 10,000 rpm for 15min. Supernatant was discarded and the cell pellet was washed twice in deionized water, recovered (for 4 min at 10000 rpm at 4°C). The cell pellet was dried 24 hr at 100°C then the total bacterial cell dry weight was determined as g/L.[19][20][21]

Estimation of PHB from the Selected Isolates:

The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight.

PHB accumulation (%) = Dry weight of extracted PHB (g/L) × 100 / DCW (g/L)

Optimization of Physical Parameters for Maximum PHB Production:

Different factors affecting PHB production by the selected bacterial isolates were studied.

Effect of different pH on the production of PHB by the selected isolates:

The best isolates from quantitative screening was selected and isolates Azo 6, Azo 9and Azo 28 were grown in the conical flask (250 ml) containing 100 ml of MSM media. The medium was prepared with different pH ranging from (6, 7and 8) and the inoculated flasks were incubated at 30°C at 150 rpm for 48 hrs and PHB was quantified.

Effect of different temperature on the production of PHB by the selected isolates:

The MSM media was prepared and the pH was adjusted to 7.2. Each bacterial isolates Azo 6, Azo 9 and Azo 28 were grown in conical flask (250 ml) containing 100 ml of sterilized medium. The cultures

were incubated on a rotary shaker at 20° 30° and 40°C at 150 rpm for 48 hrs and PHB was quantified.

Optimization of chemical Parameters for Maximum PHB Production:

Effect of Different Carbon Sources on PHB production:

The selected bacterial isolates Azo6, Azo9and Azo28 were grown in 250 ml conical flasks containing 100 ml MSM media with different carbon sources like Glucose, Sucrose, Mannitol, Maltose and Galactose at 2% concentration. The flasks were incubated at 30°C on a rotary shaker (150 rpm) for 48 hours. After incubation, PHB produced by the isolates were quantified according to Miller, (1959)[22], Santimano et al., (2009)[23], Ghate et al., (2011)[24].

Effect of Different Nitrogen Sources on PHB Production:

The bacterial isolates Azo6, Azo9 and Azo28 were grown in 250 ml conical flasks containing 100 ml MSM media with the best carbon source, and different nitrogen sources like Ammonium sulfate, Ammonium chloride, Yeast extract and Peptone were used at a concentration of 2%. After 48 hrs of incubation at 30 C PHB yield were quantified.

RESULTS:

Qualitative screening for PHB producing *Azotobacter* spp soil isolates:

All the 28 strains showed different Sudan black absorption pattern on the minimal agar. The growth and the Sudan black absorption pattern were viewed from viable colony staining technique and reported in Table 1. These isolates showed positive for the presence of lipophilic PHB granules. The efficient PHB producing strains were selected based on qualitative screening.

Extraction and Quantification of PHB:

On the basis of qualitative screening the isolates were subjected to quantitative screening for the yield of PHB. The yield of PHB was observed on a higher scale for Azo 6 isolate as it yielded 29.17%. Azo 28 yielded 11.43% of PHB and Azo 9 gave 10.42% of PHB. All the isolates were compared with the standard strain *Azotobacter vinelandii* MTCC 124 which yielded 10.16% of PHB and it is presented in Table 2.

Optimization of Physical Parameters (pH and Temperature):

Effect of different pH on the production of PHB by the selected isolates:

Three isolates, namely Azo 6, Azo 9 and Azo 28 were subjected to different pH and their PHB yield was calculated. Optimization of the production of poly hydroxy −β-butyrate under various (6,7,and 8) were analyzed at standard temperature (37°C) The results obtained from Table 3 show that the maximum production rate was at pH 7 for all the three strains .The maximum production was observed for Azo 6 and Azo 28. The maximum production of PHB was observed for the strains Azo 6 (66.66%), Azo 28 (60.41%) under optimized duration (48 hours).Effect of pH in the medium showed a strong influence on the production of poly −β-hydroxy butyrate. The maximum production rate for all the strain were observed at pH 7.The next higher level of production was observed at pH 8 and minimum production was observed at pH 6.

Effect of different temperature on the production of PHB by the selected isolates:

The selected strains from quantitative analysis were optimized for the Poly hydroxy β butyrate production under various temperature (20°C, 30°C and 40°C) and were presented in Table 4.The strain

Azo 6 yielded 79.59% of PHB at 30º C, Azo 28 yielded 59.57% and Azo 9 yielded 53.33% of PHB. All the three isolates showed the maximum production rate at 30º C when compared with other two temperatures (20^{0}C and 40^{0}C).

Optimization of Chemical Parameters (Carbon and nitrogen source):

Effect of different carbon sources on the yield of PHB:

Study on the effect on different carbon sources (at 2% concentration) on the yield of PHB revealed that all the three isolates gave good yield on the utilization of the simple sugar glucose than with other carbon sources. Table 5 shows that Azo 6 was able to yield higher percentage of PHB with both sucrose and mannitol as carbon source. Azo 9 gave a yield of 50% PHB with sucrose as carbon source. Azo 28 gave better yield with mannitol (60.66%) followed by sucrose (59.32%).

Effect of different nitrogen sources on the yield of PHB:

Study on the effect on different nitrogen sources (at 2% concentration) on the yield of PHB revealed that all the three isolates gave good yield on the utilization of ammonium sulphate than with other nitrogen sources. Azo 6 was able to yield higher percentage of PHB with ammonium sulphate, ammonium chloride and yeast extract as nitrogen source. Azo 9 gave a yield higher only with ammonium sulphate. Azo 28 gave better yield with ammonium sulphate (61.14%) and peptone (40.47%). (Table 6)

Table 1: Qualitative Screening for PHB Producing *Azotobacter* spp., soil isolates

Isolates	Growth on MSM
Azotobacter vinelandii MTCC 124	+++
Azo1	+
Azo 2	+
Azo 3	+
Azo 4	++
Azo 5	++
Azo 6	**+++**
Azo 7	+
Azo 8	++
Azo 9	**+++**
Azo 10	+
Azo 11	**+++**
Azo 12	+
Azo 13	**+++**
Azo 14	+
Azo 15	+
Azo 16	++
Azo 17	**+++**
Azo 18	++
Azo 19	+
Azo 20	+
Azo 21	**+++**
Azo 22	**+++**
Azo 23	+
Azo 24	+
Azo 25	++
Azo 26	+
Azo 27	+
Azo 28	**+++**

Growth pattern (+++) seen on minimal agar plate showed the maximum absorption Growth pattern (++) showed moderate Sudan black absorption and Growth pattern (+) showed less Sudan black absorption.

Table 2: Quantification of PHB by the selected *Azotobacter* spp., soil isolates

Code No. of isolate	DCW (g / L)	PHB (g/L)	Yield % of PHB
Azo 6	5.21 ± 0.13	1.52±0.12	29.17
Azo 9	5.37±0.12	0.56±0.11	10.42
Azo 11	5.26±0.15	0.53±0.11	10.07
Azo 13	5.42±0.18	0.45±0.12	8.30
Azo 17	5.15 ±0.11	0.40±0.09	7.76
Azo 21	5.31±0.17	0.50±0.11	9.41
Azo 22	5.40±0.14	0.42±0.09	7.77
Azo 28	5.51±0.24	0.63±0.15	11.43
Azotobacter vinelandii MTCC 124	5.61±0.12	0.57±0.14	10.16

DCW: Dry cell weight PHB: polyhydroxybutyrate

TABLE 3: Effect of different pH on the production of PHB by the selected isolates

ISOLATE	pH 6			pH 7			pH 8		
	DCW (g/L)	PHB (g/L)	YIELD %	DCW (g/L)	PHB (g/L)	YIELD %	DCW (g/L)	PHB (g/L)	YIELD %
AZO 6	1.7±0.11	0.7±0.12	41.17	5.4±0.12	3.6±0.12	**66.66**	1.4±0.12	0.8±0.12	57.14
AZO 9	1.2±0.13	0.5±0.11	41.66	5.1±0.12	3.0±0.12	58.82	0.9±0.13	0.4±0.11	44.44
AZO 28	1.6±0.15	0.64±0.13	40	4.9±0.13	2.9±0.14	**60.41**	1.1±0.12	0.6±0.11	54.54

TABLE 4: Effect of different temperature on the production of PHB by the selected isolates

ISOLATE	20° C			30° C			40° C		
	DCW (g/L)	PHB (g/L)	YIELD %	DCW (g/L)	PHB (g/L)	YIELD %	DCW (g/L)	PHB (g/L)	YIELD %
AZO 6	2.12±0.2	1.4±0.11	66	4.9±0.2	3.9±0.11	**79.59**	2.11±0.11	0.3±0.08	14.21
AZO 9	2.11±0.11	0.4±0.11	18.95	4.5±0.3	2.4±0.12	53.33	1.82±0.12	0±0	0
AZO 28	2.05±0.33	0.8±0.12	39.02	4.7±0.11	2.8±0.14	**59.57**	2.04±0.13	0.2±0.06	9.80

Table 5: Effect of 2% carbon sources on the production of PHB

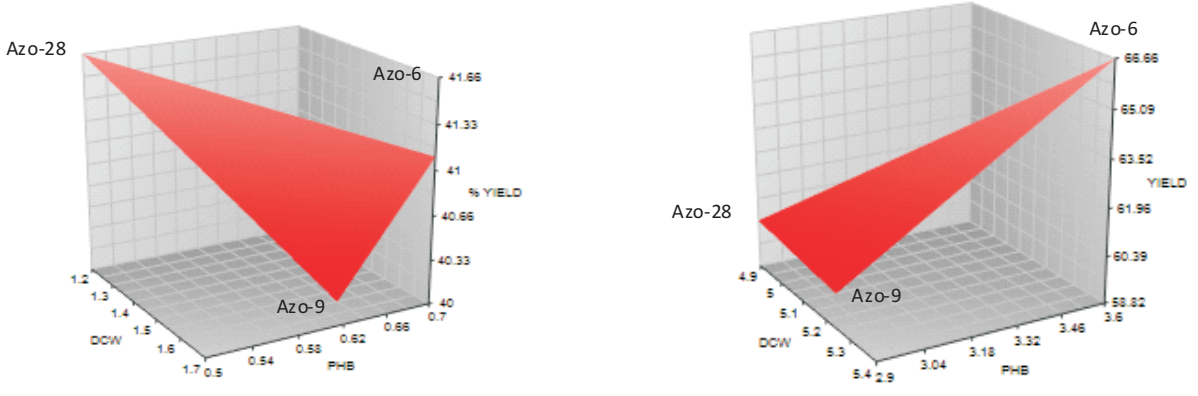
ISOLATE	Azo 6			Azo 9			Azo 28		
Carbon sources	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**
Glucose	2.4±0.12	1.47±0.16	**61.25**	2.5±0.16	1.43±0.11	**57.2**	2.52±0.15	1.36±0.12	53.96
Sucrose	3.7±0.11	3.03±0.12	**81.89**	2.10±0.11	1.05±0.15	50	3.54±0.15	2.10±0.13	**59.32**
Mannitol	3.4±0.12	2.98±0.13	**87.64**	2.98±0.12	0.84±0.18	28.18	3.33±0.15	2.02±0.14	**60.66**
Maltose	2.85±0.18	0.98±0.15	34.38	2.14±0.15	0.62±0.18	28.97	2.16±0.16	0.84±0.06	38.88
Galactose	2.30±0.16	0.81±0.15	35.21	2.42±0.13	0.45±0.14	18.59	2.41±0.14	0.63±0.04	26.14

Table 6: Effect of 2% nitrogen sources on the production of PHB

ISOLATE	Azo 6			Azo 9			Azo 28		
Nitrogen sources	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**	**DCW (g/L)**	**PHB (g/L)**	**YIELD %**
Ammonium sulphate	3.74±0.12	2.58±0.08	**68.98**	3.68±0.15	1.96±0.15	53.26	3.14±0.05	1.92±0.11	**61.14**
Ammonium chrolide	3.82±0.11	1.9±0.09	49.73	3.10±0.12	1.22±0.12	39.35	3.18±0.12	1.15±0.16	36.16
Yeast extract	2.6±0.14	1.06±0.2	40.76	3.18±0.11	1.10±0.09	34.59	2.85±0.08	1.09±0.18	38.24
Peptone	3.1±0.10	1.10±0.14	35.48	2.9±0.05	1.15±0.10	39.65	2.52±0.11	1.02±0.05	40.47

Fig 1: Effect of different pH on the production of PHB by the selected isolates

a) pH 6 **b) pH 7**



c) pH 8

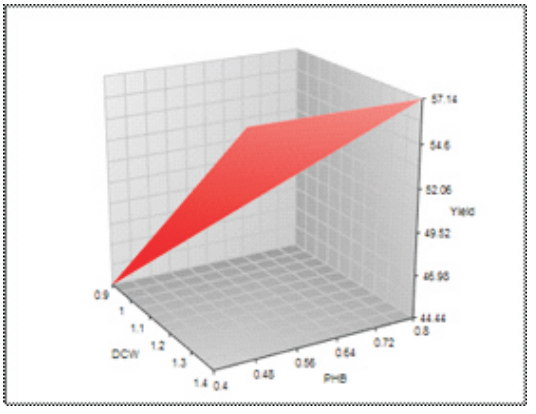
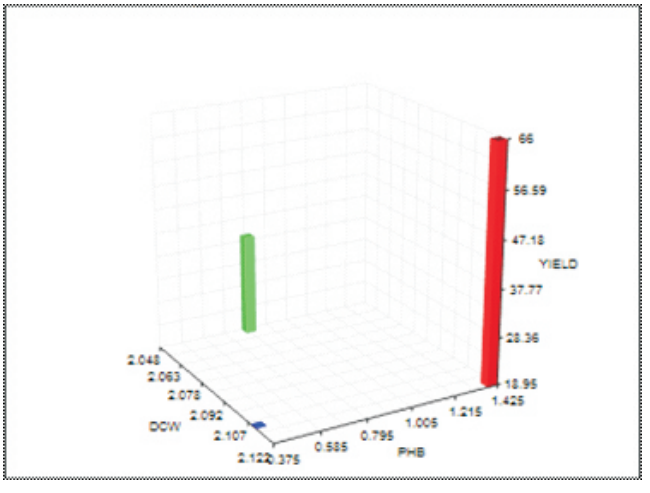
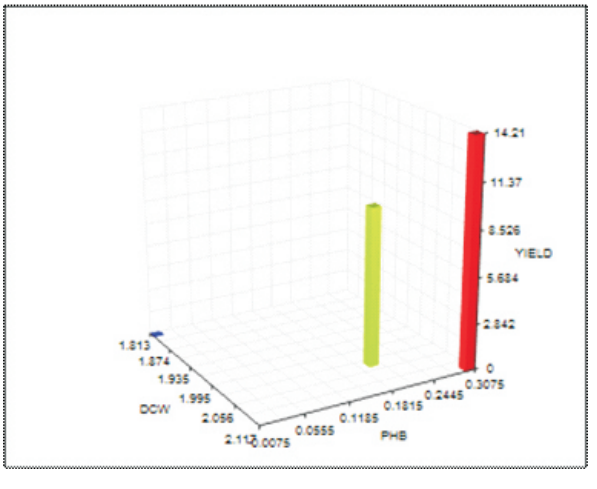


Fig 2 : Effect of different temperature on the production of PHB by the selected isolates

a) PHB yield at 20° C



b) PHB yield at 30° C



c) PHB yield at 40° C

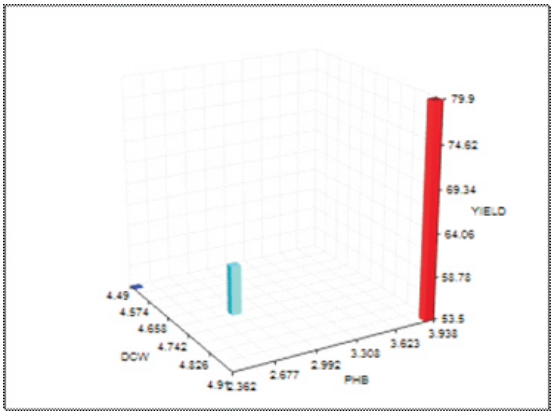


Fig 3: Effect of different carbon sources on the production of PHB by Azo 6

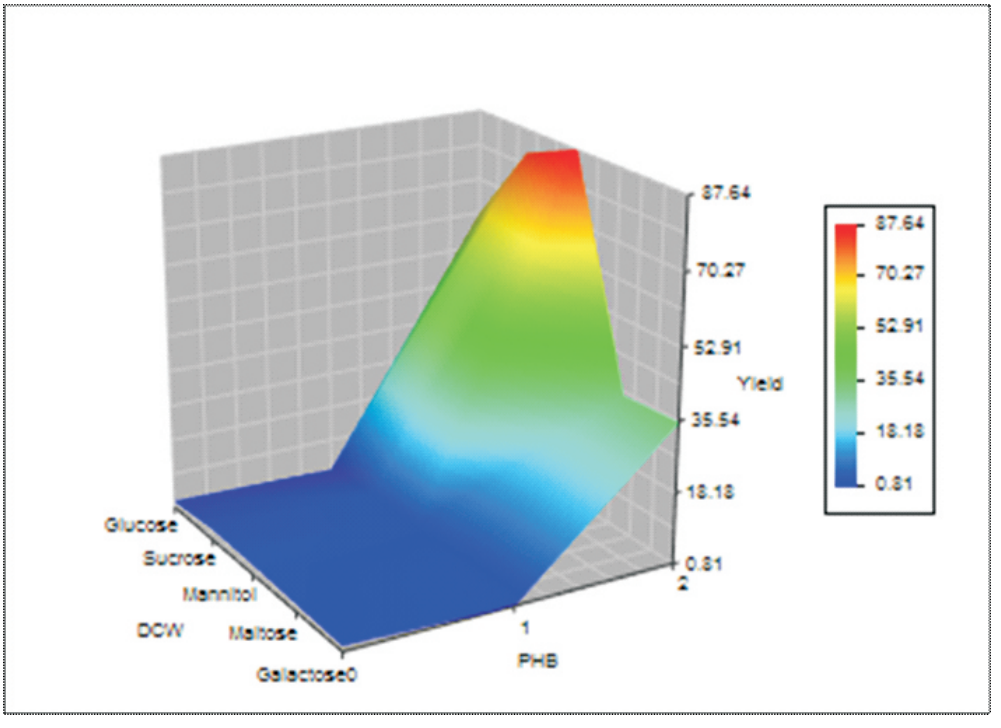


Fig 4: Effect of different carbon sources on the production of PHB by Azo 9

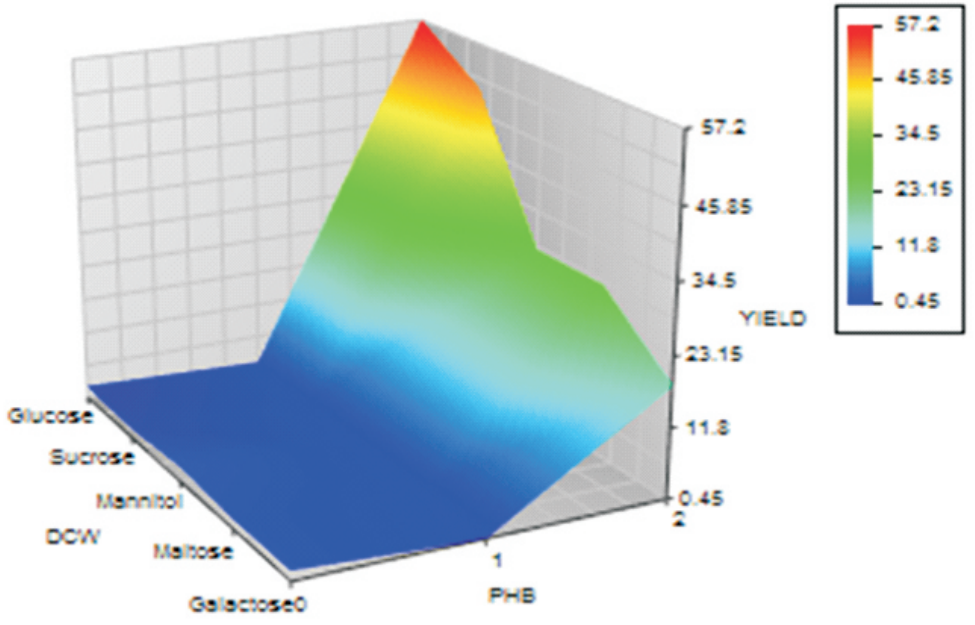


Fig 5:Effect of different carbon sources on the production of PHB by Azo 28

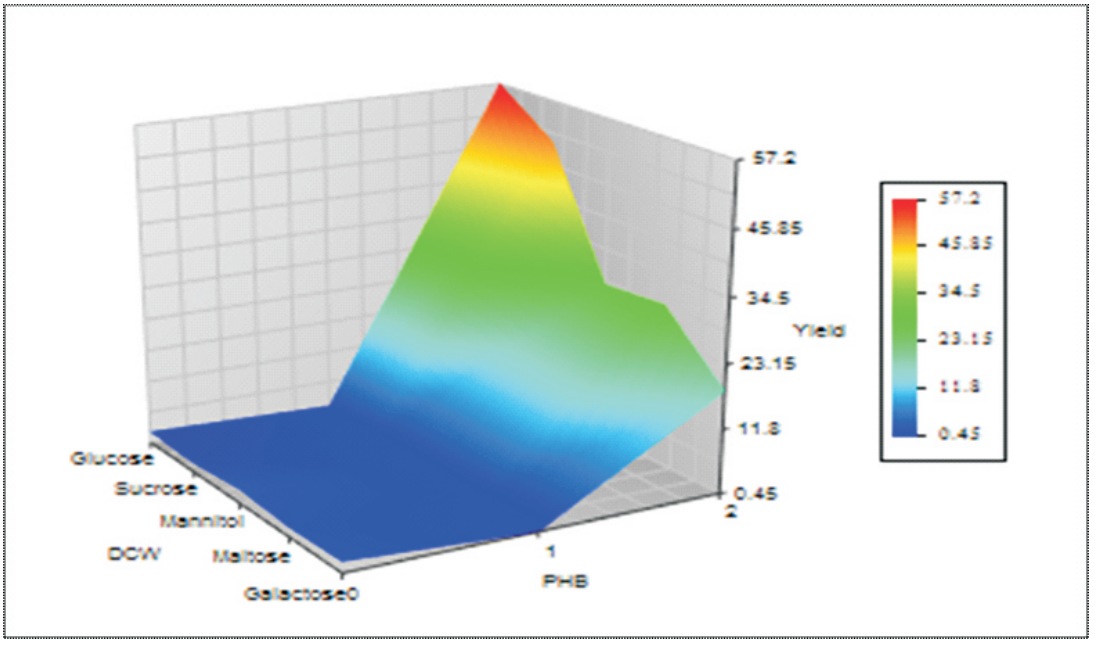


Fig 6:Effect of different nitrogen sources on the production of PHB by Azo 6

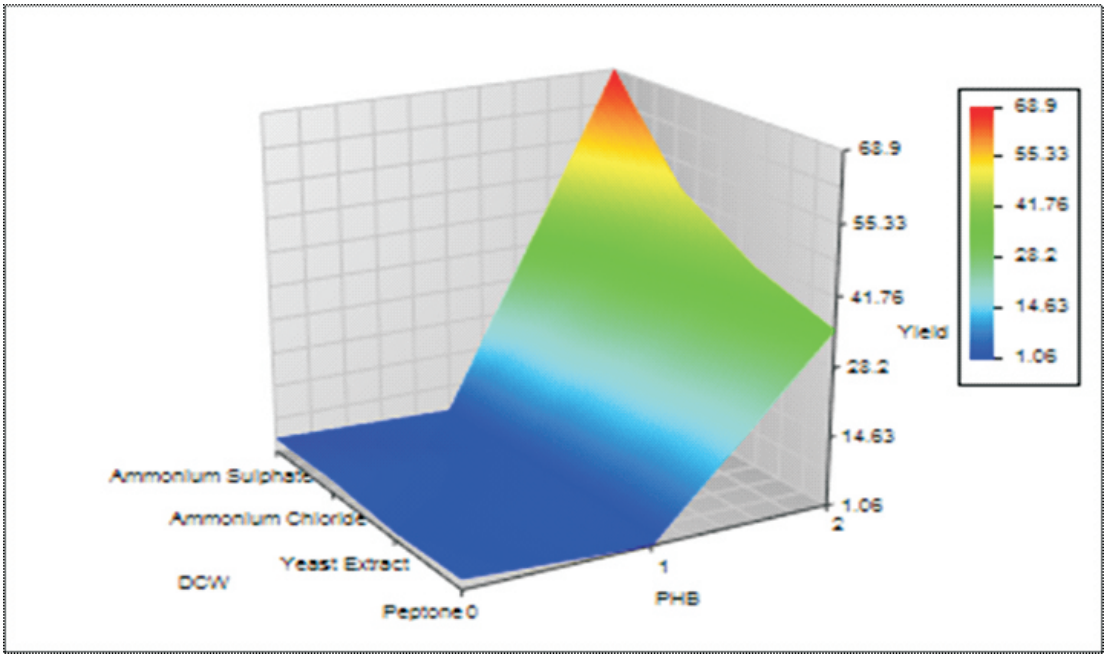


Fig 7: Effect of different nitrogen sources on the production of PHB by Azo 9

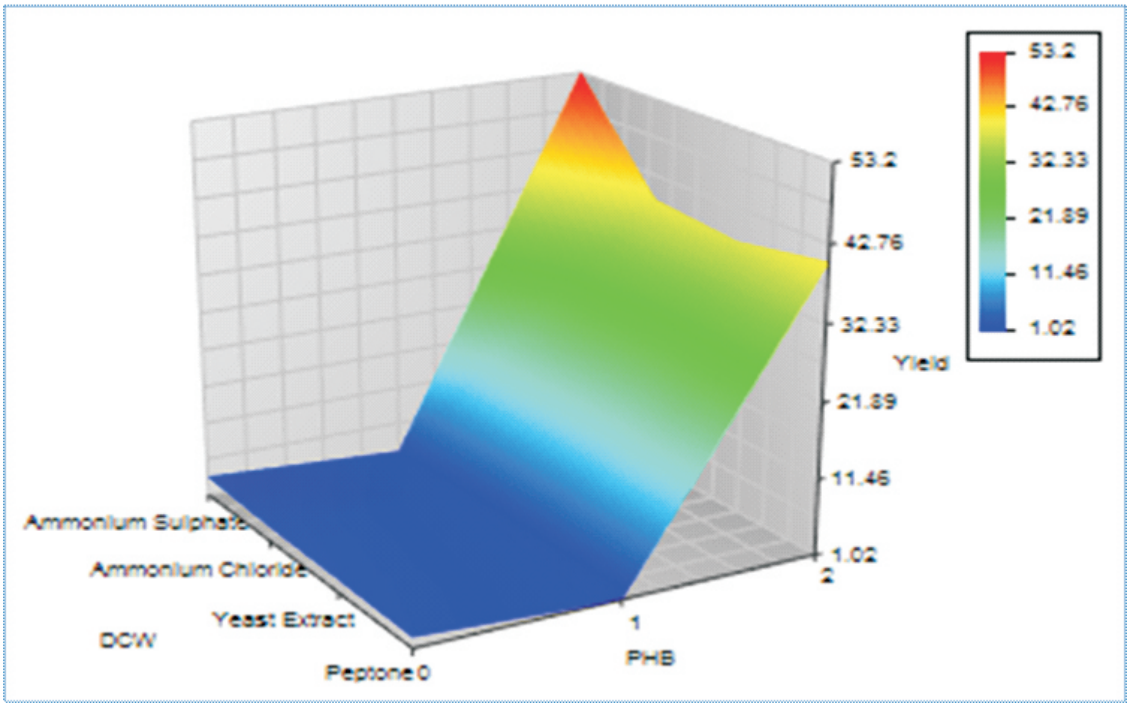
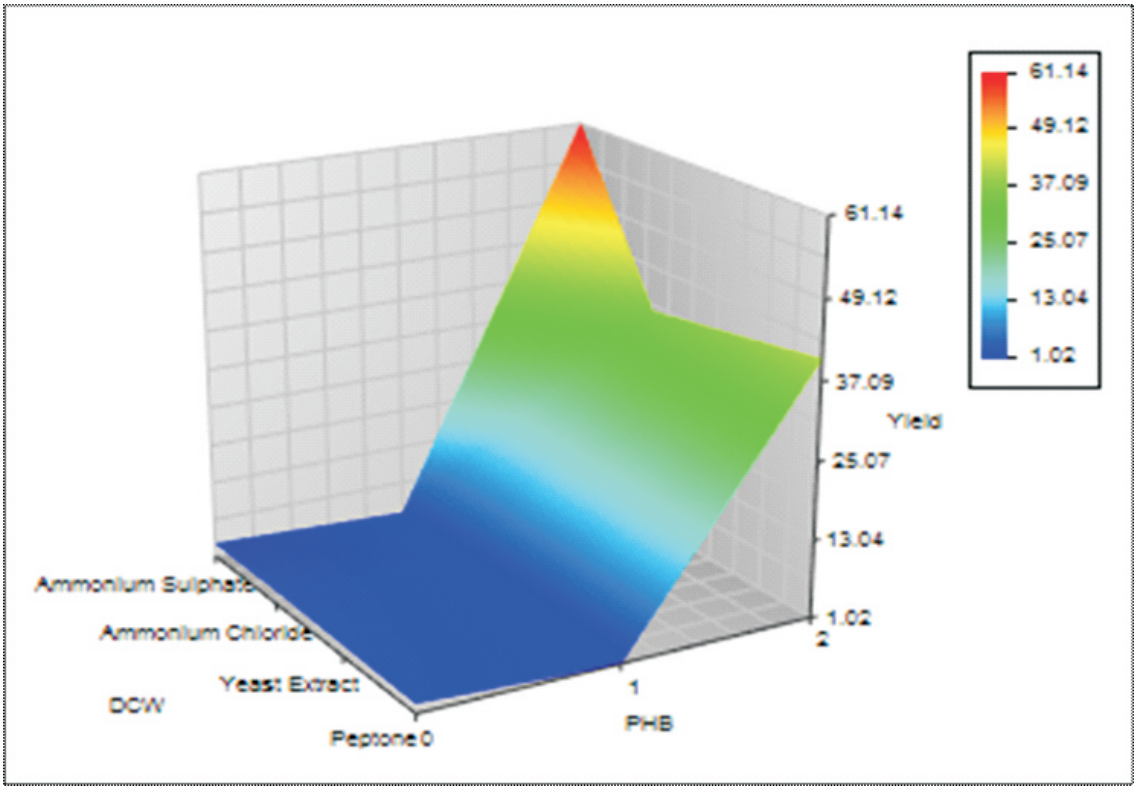


Fig 8: Effect of different nitrogen sources on the production of PHB by Azo 28



DISCUSSION

Poly-β- hydroxybutyrates is a common reserve material in prokaryotes, which is present in both Gram positive and Gram negative bacteria. PHB is a polymer of D(-)-β-hydroxy butyrate and had a molecular weight between 60,000 and 2,50,000. Polymer accumulation was initiated under nutrient imbalance and serve as an electron and carbon sink. PHB usually function as a carbon or energy source and is degraded under condition of stress and starvation. Poly β hydroxybutyrates (PHAs) are bacterial polymers that are formed as naturally occurring storage polyesters by a wide range of microorganisms usually under unbalanced growth conditions. It is well known that PHB synthesis is closely connected with the energy requirements of the cell. Many nitrogen-fixing microorganisms can synthesize PHB in considerable quantity, because its synthesis and consumption are closely connected with such energy - intensive process as the biological fixation of molecular nitrogen. PHB synthesis depends on a number of conditions including the nature of carbon and nitrogen sources utilized, on their concentration ratio in the medium, on partial oxygen pressure and so on. In considering PHB synthesis by Azotobacter spp., it should be noted that this property is fully inherent in all Azotobacter species.

Qualitative screening for PHB production was performed on all the 28 isolates and the isolates Azo 6, Azo 9, Azo 11, Azo 13, Azo 17, Azo 21, Azo 22, Azo 28 showed maximum absorption pattern with Sudan black B staining.

Based on the qualitative screening, eight isolates namely Azo 6, Azo 9, Azo 11, Azo 13, Azo 17, Azo 21, Azo 22 and Azo 28 were quantitated for PHB yield along with the reference strain A.vinelandii. Azo 6 produced the highest PHB of 29.17%. Azo 28 yielded 11.43% of PHB and Azo 9 gave 10.42% of PHB greater than the reference strain cut off.

Optimization of physical parameters like pH and temperature revealed that the optimum pH of 7 and Temperature of 30ºC is ambient for the bacteria to produce PHB.

The microbial production of PHB is dependent on carbon and nitrogen sources. Various nitrogen-rich media containing casein hydrolysate, yeast extract, tryptone, casamino acids, corn steep liquor and collagen hydrolysate [25][26][27][28] have been used for PHB production. Carbon-substrate rich media such as molasses, whey, hemicelluloses, palm oil, starch, glucose, fructose, sucrose, maltose, gluconate or glycerol accumulates PHB in bacterial cells [29][30][31][32][33] and have been used as substrates for PHB production. However, unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract, also supported PHB formation, obtaining yields of PHB comparable to, even better than the refined sugars.

The present study investigated on five different carbon sources (Glucose, Sucrose, Mannitol, Maltose and Galactose) and four different nitrogen sources (Peptone, Ammonium chloride, Ammonium sulphate and Yeast extract). It was observed that the yield of PHB from Ammonium sulphate were much higher than the other nitrogen sources. The strains Azo 6 and Azo 28 showed the maximum production rate when compared to the standard strains. Azo 6 gave a higher percentage yield of 87.64% PHB with Mannitol as carbon source and 81.89% with sucrose as carbon source. Similarly, Ammonium sulphate utilization gave a yield of 68.98% of PHB .The similar results were supported by Wu et al., (2001)[34], the highest level of PHB accumulation was observed in the media with Protease peptone as nitrogen sources in Bacillus subtilis 25 (78.69%) and in Bacillus megaterium 12 (77.00%). Page, (1992)[35] reported, that the PHB production in a variety of commercially available complex nitrogen sources (fish peptone, protease peptone, yeast extract, casitone, phytone and tryptone) increased the yield of PHB produced by Azotobacter vinelandii UWD strain. Mercan et al., (2002)[36] investigated the effect of different nitrogen and carbon sources and PHB production in two strains of Rhizobium species and the strains produced less PHB in yeast extract mannitol (YEM) broth media with different carbon (glucose, sucrose, arabinose) and nitrogen (L-Cysteine, L- glycine, DL-tryptophan, protease peptone, potassium nitrate) sources, while the highest level of PHB accumulation was observed in the media with L-cysteine and L-glycine. According to Khanafari et al., (2006)[37] study, the production of PHB in Azotobacter chroococcum 1735 which produce the maximum PHB percentage was determined in Meat extract as a nitrogen source. While the percentage yield of PHB in the strain was lower with different nitrogen source in Mannitol broth, the highest level of PHB accumulation was observed in the media with Meat extract as nitrogen source.

Molecular structure of PHB doen't depend on the features of the strain and conditions of carbon nutrition of microorganisms producing PHB [38]. Most of the bacteria which produce PHB are nitrogen-fixing microorganisms. The Azotobacter species fix the molecular nitrogen and have the capacity to accumulate poly -β- hydroxybutyrates when they are grown on different carbon sources, including sucrose media[39]. The cysts formed during adverse conditions germinate under favourable conditions to give vegetative cells, produce polysaccharides. These bacteria utilize atmospheric nitrogen (N2) for their cell protein synthesis. Literature support that most of the nitrogen fixing cells have a low PHB content and the addition of nitrogen spares the need for nitrogen fixation and the respiratory protection of the oxygen labile nitrogenase complex and thus allows the reducing power and Acetyl Co A derived from active sugar metabolism to be used for PHB production.Similar results were obtained by present research. Boem Soo kim and Ho Nam Chang, (1998)[40] reported the ability of Azotobacter chroococcum to produce PHB from starch and that the PHB content increased up to 74% of the dry cell weight with increasing culture volume. Okon and Itzigsohn (1992)[41] found PHB accumulation to the level of 70 per cent of cell dry weight with strains possessing high nitrogen fixation,

establishment and survival.

CONCLUSION:

The soil isolates Azo 6 and Azo 28 produced a higher percentage of PHB. These organisms can be manipulated at the genetic level to increase this biopolymer production as it would help in making biodegradable plastics.

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