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PRODUCTION OF POLY B-HYDROXY BUTYRATE FROM DISTILLERY SPENT WASH USING MUTANT *Azotobacter Vinelandii*

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ABSTRACT:

Poly Hydroxy butyrate are of human interest due to there ability to act as an important constituent of bioplastic which can effectively replace polyethylene, a non biodegradable plastic. Distillery spent wash (DSW) is an industrial waste which have high COD levels but consist of useful minerals that can be utilized for PHB production. Azotobacter vinelandii ATCC 12837 and ATCC 13705 were utilized to develop Azotobacter vinelandii UWD; a hyper PHB producing mutant, via NTG mutagenesis. This strain was grown in fed batch fermenter containing diluted DSW
(1 -7%) supplemented with 5% glucose and ammonium acetate as carbon source and nitrogen source respectively. The transformation efficiency for Rif + colonies was 9 × 10 -5 which was good enough to select the hyper PHB producing mutants after incubation of 5 days. The amount of PHB produced in various dilution of spent wash was highest at 4% spent wash of 40 g/L (92%w/w PHB). The glucose consumption was 89%w/w at 4% DSW. The dry cell weight at 4% DSW was >23.4 gm/L. The elevated COD levels (1100 mg/L) of DSW were reduced to 200 mg/L after fermentation was completed. The spent wash which was considered to be a waste and environmental pollutant can be hence utilized for bioplastic production

KEYWORDS: poly β-Hydroxy butyrate, Azotobacter vinelandii, spent wash, fed batch fermentation

[I] INTRODUCTION

Properties And Scope Of PHB

Poly ß hydroxybutyrate is a high molecular weight polyester with monomer- R 3 HBA (hydroxyl butyric acid) [1] which is accumulated
as a storage carbon in many species of bacteria and is a biodegradable crystalline thermoplastic [2]. Polyhydroxy alcanoates like poly ß hydroxybutyrate / Polyoctanoates are accumulated as inclusion bodies or PHA granules during high carbon feeding rates [2-5]PHA exists in two forms PHAscl and PHAmcl the PHB is a PHAscl with 4 numbered carbon chain [6]. The PHA is storage compound accumulated in several bacterial species during unbalanced growth conditions [35,2] PHB is the most abundant type of PHA out of 140 existing different PHA's [36].
The PHA are generally water insoluble, elastomeric, non-toxic, enanciomerically pure, biocompatible

bioplastic [7-9] this class of polymer is completely biodegradable which can be digested and metabolized by a wide variety of bacteria and fungi in living body, soil or in other environment. Recently it has been observed that PHB and PVA (poly vinyl alcohol) blends were more biodegradable and stable as compared to pure plastics [10]. Remaining types of degradable plastics include starch and polyester based bioplastic, which is already commercially produced under brand name Nova Mont in Italy, Sky Green-Korea ,Bionolle – Japan [10]. Since ester bonds are thermally weak so polyesters are easily hydrolyzed in hot and humid condition. The co-polymer PHB/HV(Hydroxy valerate) BIOPOL/BIOMER[11], PHB/HH(Hydroxy Hexanoate) ,PHB/PEO(Poly ethylene oxide)[10] were found to be less brittle than homopolymeric forms. Bio-plastic tensile strength has been studied up to 400 Mpa [12]. Development and growth of petrochemical industry has caused lowering in cost of oil derived polymers than their natural counterparts. Despite this natural polymers are extensively used and have many advantages over synthetic rivals [13], melting point of PHB is 180˚C. In general PHB properties are comparable with poly propylene due to similar melting point, crystalline nature, glass rubber transition temperature [2,14,8], it is highly brittle, low solvent resistant but high ultraviolet weathering resistant the molecular weight is 183200 Daltons.

Blends of PHB /Valerate (3 Hydroxy Pentanoate), PHB/PVB (Poly Vinyl Butrylene), PHB/poly p-dioanone were also studied for similar properties [15]. The blending of PHB has shown to reduce crystalline nature, melting point and stiffness which are beneficial for increasing flexibility as bioplastic. PHB is optically active, chiral carbon in D (-) configuration rotates the plane of polarized light passing through it making it applicable in chromatography for optically active sample separation [16,14].

It is a constituent of human blood (D (-) Hydroxy butyrate), used as replacement of glucose (oral/intravenous) [17], truly biodegradable and completely converted to CO2 and energy by microorganisms like bacteria ,fungi, algae. The blends of PHB includes some blends being completely biodegradable and some partially biodegradable [37].

Rate of degradation depends on several factors i.e. temperature, BOD, Oxygen supply etc. Rate of PHB oxidation is negligible in moist air hence its shelf life is higher. All these attractive properties of PHB can be used to replace conventional non biodegradable polymer like polyethylene and poly propylene [37]

The PHA is synthesized by many microorganisms such as Cupriavidus necator, Alcalegenes latus, Aeromonas hydrophila, Pseudomonas putida and Bacillus species [18,19].

The most proffered organism for PHB production is Alcalegenes eutrophus which was first isolated in north Germany soils.

Production of PHB in A.vinelandii:

Azotobacteriaceae members have been extensively studied for storage of PHB [38].

Azotobacter vinelandii is obligate aerobe able to fix nitrogen, biosynthesize alginate and poly hydroxyl butyrate [20].Azotobacter species and others microorganisms are known for production of alginate, siderophores and PHB where alginate and PHB biosynthesis are inversely correlated. The alginate production will reduce in absence of limiting nutrient (Nitrogen, Oxygen, Phosphate) resulting in accumulation of carbon in form of PHA granules. Contrary alginate production increases in excess carbon feeding during favorable conditions. During stress condition Azotobacter species will move towards starvation and assimilate internal carbon reservoirs as PHB and forms cysts [21,22].

PHB production is dependent on NADH pathway; precursor for PHB is acetyl co-A which is produced by ED pathway. During unbalanced growth entry of acetyl co-A is restricted in TCA cycle due to the inhibition of citrate synthase enzyme caused by elevated levels of NADH [39,40]. NADH can be oxidized to NAD+, eliminating this growth inhibition, by the action of acetoacetyl CoA reductase and the polymerization of acetoacetyl

CoA into PHB, thus accumulated acetyl CoA is directed for PHB synthesis. PHB production increases (3 – 10 folds) in mutant Azotobacter vinelandii incapable to synthesize alginate [23,24]. PHB gene cluster phbBAC is involved in coding three enzymes for PHB synthesis: phbA-ketothiolase, phbB-Acetoacetyle CoA reductase and phbC-PHB synthase respectively. The studies of PHB production has also been performed in most proffered E.Coli cells (recombinant) in which the PHB producing genes from host organism were introduced [41,25]. Though there are very limited studies of PHB production using Azotobacter vinelandii at bioreactor level (lab and pilot scale both) [42,26,27] also there are no reports of scale up of A.vinelandii in PHB production. Large scale production of bacterial PHA possesses a number of problems cost of fermentation, extraction of polymer from cell

A potential cost saving method is offered by use of A. vinelandii UWD mutant for PHA production [43]. Since A. vinelandii nutritional requirements are very less so it can be cultivated on some low cost substrates i.e. beet molasses, swine wastes. Spent wash may be used as one of the cheapest sources for large scale bioprocess, 295 distilleries in India producing 3198 million liters of ethanol per year. Production of 1 liter of ethanol generates 15 liter of dark brown, highly acidic spent wash. It means 4.5 lakh liters of waste water in form of spent wash is produced and dumped in water bodies creating serious environmental concern [nariphaltan.virtualave.net/detox.pdf].

A vinelandii wild-type (OP) have been identified and registered in American Type Culture Collection under accession number ATCC 13705. The University of Wisconsin has developed their own strain (UW) of A. vinelandii and registered as ATCC 12837.

[II] MATERIALS AND METHODS Growth, subculture and mutagenesis:

Azotobacter strain (UW) ATCC 12837 [National Collection of Industrial Microorganism(NCIL) ,National Chemical Laboratory- Pune] and ATCC 13705 OP(LGC promochem - Bangalore), sub cultured and maintained on modified Burks media in appropriate formulations containing grams liter^{-1} of Magnesium sulphate 0.2, Dipotassium phosphate 0.8, Monopotassium phosphate 0.2, Calcium sulphate 0.130,Ferric chloride .00145, Sodium molybdate 0.000253 , Sucrose 10.0 ,glucose 10.0 and agar 10.0 respectively pH -7.2.,poured in 6 petriplates and was incubated at 30 C [28]

After 24 hours of incubation period, the colonies (ATCC 12837and ATCC 13705) were collected and transferred to two sterile test tubes containing 10 mL of Burk's buffer. After attaining exponential phase (2×106 cells/ml) the culture tube of ATCC 12837 was supplemented with 100 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at pH 7.2, for 20-30 minutes in dark without shaker . After incubation, 50 µl of culture was inoculated in petriplate containing sterile Burk medium supplemented with 1% glucose, 1.8% agar and 20 µg/ml Rifampin. These plates were incubated at 30˚C for another 24 hours.

Transformation and transformants selection:

After 24 hours the colonies were selected as a Rif $^{+}$ mutant 113 of ATCC 12837, transferred in 10 mL Burks buffer, grown and used as donor cells. DNA was isolated from encapsulated Rif + ATCC 12837, by cell lyses method in 15mM Nacl/ Na-citrate buffer with SDS (0.5% freshly prepared) at pH-7. After incubation at 55˚C for 45 minutes the lysate DNA was collected by centrifugation and assayed for purity. Crude lysate DNA was precipitated in 1:3 water and ethanol (95 %) v/v, centrifuged and re-suspended in 20 mM saline. The entire 10 ml of ATCC 13705 culture was aseptically inoculated in 100 ml of Burks media and incubated for 24 hours till late exponential phase is achieved. Cell pellet (100-200 µl) of ATCC 13705 (capsule negative) were collected (3×108 cells/ml) by centrifugation at 3000 rpm for 10 minutes on 4˚C were obtained in sterile eppendorf containing 500µl of Burks buffer (supplemented with 15mM Ammonium Acetate (AA), 8.5mM MgSO4) followed by 2µg lysate DNA was added aseptically and incubated at 30˚C for 30 minutes. It will increase transformation efficiency. Pre-incubation of the said transformants

on non selective medium (Burks medium) was performed for 12 hours followed by transferring colonies on petriplates containing agar and Rifampin (20 μg) supplemented Burks media. After incubation for 3-4 days the sectored white colonies were scraped off and re-streaked on modified Burks media that resulted in development of milky white colonies(UWD)[28]. PHB granules have been observed in the cytoplasm under research microscope.

Shake flask culture and Fed batch Fermentation:

These mutant PHB producing colonies [28], were sub cultured and maintained in modified Burks media containing 2.0 mg L-1 Fecl3 and 1.5 % Agar for 2-3 weeks at 28˚C. The colonies of UWD were transferred into 20 ml sterile modified Burk's media without agar. After 24 hours the 10 ml of this culture was added aseptically in to 100 ml of sterile modified Burk's media and further incubated for 24 hours. Log phase culture of UWD was used as inoculums in shaker flask. Ten percent of starter culture was used as seed culture for fed batch fermentation (New Brunswick - USA). Cell culture monitoring and control was performed by computer (Advanced Fermentation Software New Brunswick science), External heating blanket and immersed stainless steel cooling coil were used to maintain convective temperature at 28°C. Magnetic Drive was used to control agitator shaft rotations at 250-300 rpm. Three Rushton style standard impellor blades mounted on agitator shaft were used for churning spent wash media. pH was maintained at 7.0 using 1 M NaOH with proportional–integral–derivative controller (PID), antifoaming agent provided by sigma was used to control excess foam. The spent wash provided from united distilleries, Jaipur was collected in polycarboryl container; properly sealed and stored at 4°C for further analysis. The physico-chemical properties were analyzed by standard methods of APHA [29].

The effect of various carbon sources, spent wash with several dilutions supplemented with glucose as growth substrate has been studied in shaker flasks and after optimization the effect on production of PHB was investigated in a 1.3 L Fermentor. Spent wash was sterilized, filtered and centrifuged at 6000 rpm for 20 minutes to remove solid aggregates. At initial stage concentration of spent wash (700ml) in the fermenter was 4 % (v/v) in distilled water which was then feed supplied 350 ml of 1:1 diluted spent wash (with glucose 50%w/v) in distilled water delivered at a rate of 17.5 ml /hour for 15-20 hours. The sugar concentration was maintained at 5 % thereafter. The addition of KH_2Po_4-K_2HPo_4 (0.5M) was performed at peak 12 Hours which maintained the required concentration. Initial concentration of AA in the fermenter was 20 mM which was maintained constant by feeding 0.25 M AA at rate of 15 mL /hour. Air flow of 2 L/minute was maintained antifoaming agent (sigma) was added to control the foaming at regular intervals. The repetitive sampling was performed via sampler

Screening and detection of PHB inclusions in Azotobacter vinelandii:

Loop full of culture from fermenter was transferred at regular intervals on a glass slide, heat fixed and stained with Sudan black dye (in ethanol solvent) for 10 minutes. Addition of xylene followed by saffranine and organisms was observed under oil immersion microscopy. Cells were extracted with commercial bleach containing 5.25% sodium hypochlorite at 37˚C for 1 hour as described by Law and Slepecky. The acetone insoluble milky white residue remaining at the end of extraction procedure was dissolved in chloroform and was converted to crotonic acid by heating at 90°C in concentrated sulphuric acid for 1 hour. PHB was detected by UV Spectrophotometry (λmax. 208 nm). PHB content was defined as the percentage of PHB weight to dry cell weight. Residual biomass was equal to dry cell weight minus PHB weight.

[III] RESULTS AND DESCUSSION

phb production was carried out in fed batch fermentation by A.vinelandii UWD. Glucose in diluted DSW and AA were employed to provide the desire concentrations of glucose and nitrogen in the spent wash fermentation medium. The optimum bacterial growth leads to PHB accumulation which is dependent on the composition of the fermentation medium. the strain UWD was developed by

transforming recipient strain ATCC 13705 with donor strain ATCC 12837–strain 113. When the transformation of A.vinelandii UW OP ATCC 13705 was carried out using DNA of strain 113, it was observed that ATCC 13705 produced enough Rif $^{+}$ colonies when grown in Burks media. After 5 days 10 % Rif $^{+}$ colonies transformed in to milky white colonies. The transformation efficiency for formation of Rif $^{+}$ colonies was observed to be 9×10^{-5}. The growth rate of A.vinelandii UWD was observed with prolonged lag phase followed by log phase at 620 nm [Figure 1].

The exponential phase was achieved after 15 – 17 hours of incubation in spent wash-glucose-ammonium acetate media.

the amount of PHB produced was tested at various dilutions of spent wash (Table 1), supplemented with ammonium acetate was 68%(w/w) at 1 % DSW, 75 %(w/w) at 2 % DSW,89%(w/w) at 3 % DSW,92%(w/w) at 4% DSW, and further as shown in Figure 1. the highest PHB production was observed at 4 % spent wash dilution, it may be due to two important reasons first due to optimal supply of nutrients required by A vinelandii [30] and second increase in spent wash concentration enhances cod levels of the diluted spent wash which interferes with normal growth of A.vinelandii. the glucose consumption of mutant a. vinelandii was 89% (w/w) at 4% spent wash which is exceptionally higher as compared to other dilutions (Figure 1).the maximum dry cells weight at various dilutions of spent wash was observed highest of 23.4 Gm/L at 4 % DSW dilution, 7.5 Gm/L at 1%DSW, 14.3 Gm/ L at 2%DSW, 18.5 Gm/L at 3 percent

S.No.	components of DSW	Concentration in undiluted spent wash(mg/L)	Concentration after dilution: Mg/L in 5 % glucose sol. w/v and AA(Amonium acetate)					
			1 %	2%	3%	4%	5%	6%
1	pH	6.23	Maintained at 6.5 units using 1M NaOH					
2	Total Solids	30300	303	605	910	1212	1516	1819
3	Total Dissolved Solids	22356	224	448	672	896	1120	1344
4	Total Suspended Solids	8723	87.2	174	262	349	436	523
5	Settleable Solids	8432	84.3	169	253	337	421	506
6	COD	28693	287	574	861	1148	1435	1722
7	BOD	14982	150	300	450	600	750	900
8	Carbonate	scarce	--	--	--	--	--	--
9	Bicarbonate	11990	120	240	360	480	600	720
10	Total Phosphorus	27.43	0.27	0.54	0.81	1.08	1.35	1.62
11	Total Potassium	5800	58	116	174	232	290	348
12	Calcium	883	8.83	17.6	26.4	35.3	44.1	53
13	Magnesium	832	8.32	16.6	24.9	33.2	41.6	49.9
14	Sulphate	4800	48	96	144	192	240	288
15	Sodium	523	5.23	10.4	15.6	20.9	26.1	31.3
16	Chlorides	5910	59.1	118.2	177.3	236.4	295.5	354.6
17	Iron	8.1	.081	0.162	0.243	0.324	0.405	0.486
18	Manganese	1385	13.85	27.7	41.5	55.4	69.2	83.1
19	Zinc	1.09	0.01	0.02	0.03	0.04	0.05	0.06
20	Copper	0.289	0.0028	0.005	0.008	0.011	0.014	0.016
21	Cadmium	0.032	0.0003	0.0006	0.0009	0.0012	0.0015	0.0018
22	Lead	0.14	0.0014	0.0028	0.0042	0.0056	0.0070	0.0084

TABLE 1. Components of DSW and concentration at different dilutions in 5% Glucose and 0.15 M Ammonium

DSW (Figure 2). The residual biomass values were ranged between 3.4 Gm/L at 1% dilution to 7.4 Gm/L at 4% dilution DSW. The COD level of 4 % DSW at starting was adjusted to 1148 Mg/L which drastically came down with incubation time as shown in (Figure 3). while as the values of COD observed static during initial 10 – 15 hours and then reduced exponentially with incubation time period, levels were 1100 Mg/L at

20 hrs., 980 mg/l at 25 hrs, 900 Mg/L at 30 hrs, 600 mg/l at 35 hrs, 500 Mg/L at 40 hrs, 350 Mg/L

t 45 hours and 200 mg/l at 50 hr respectively as estimated by APHA .Azotobacter vinelandii will produce PHB in diluted spent wash which is not previously studied. The reductions in cod levels of spent wash have also been observed previously with A.eutrophus [30]. the strain uwd was purified from the sectored colonies that confirmed a pattern of clonal as well as nonclonal growth as previously observed [31] the UWD cell growth increases as more cells became homozygous for the chromosome which expresses mutant NADH oxidase gene[32]. Out of large population of viable NTG mutants only limited clones of A.vinelandii with mutant chromosomes express the white phenotype.

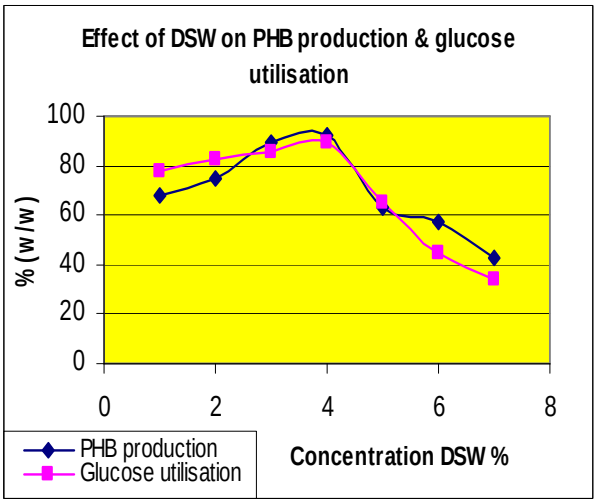

Fig. 1

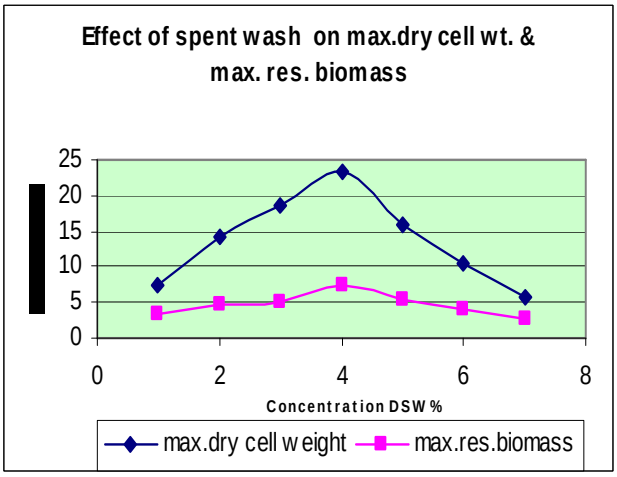

Fig. 2

This mutation is however neutral mutation which is neither useful nor harmful to A.vinelandii cells.[28] the spent wash acted as a good mineral source in diluted form for growth of A.vinelandii cells, the high cod levels were adjusted to appropriate levels using glucose solution. Distillery spent wash has already been utilized for the production of xylanase enzyme from burkholederia spp. [33] and laccase enzyme from Aspergillus heteromorphus [34]. The maximum enhancement in terms of PHB production has been observed at 4% spent wash which may be due to optimal dilution which supplied accurate concentration of minerals required by A.vinelandii.

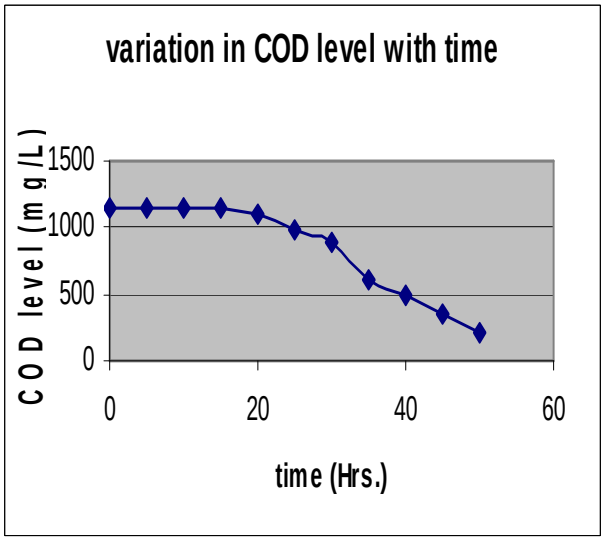

Fig. 3

The glucose gives carbon and energy source to recipient microorganisms. The ammonium acetate acts as best nitrogen source and promotes growth and PHB production [28] unlike the ammonium sulphate that results in slow PHB accumulation. The staining with Sudan black showed characteristic black colored PHB granules against pink background and PHB was detected spectrophotometrically.

CONCLUSION:

The experimental results demonstrated here are of great importance for developing economically efficient PHB production pathway. PHB production using cheap carbon sources like molasses, hydrolyzed starch, cellulose has already being

identified but no one supplied minerals regularly which were needed for any industrial production. in this study DSW has been discovered a base for PHB production and it gives regular and optimum supply of minerals when it is supplemented with glucose and ammonium acetate. Since spent wash has high mineral content and COD so it was accurately diluted and optimized before being utilized for PHB production. The DSW served two purposes, firstly it provided good combination of minerals, metal ions to A. vinelandii so that the higher production of PHB was achieved and secondly the high COD values of spent wash which is an environment pollutant were reduced to permitted values. Therefore the results of present study could be useful in improving the economy of PHB production and reducing environmental pollution caused by DSW. The economy of PHB production may be further improved by combined use of molasses and DSW as carbon and mineral source respectively. Molasses are waste generated by sugar industries and are cheap carbon source similarly the DSW is distillery waste and environmental pollutant thus major cost of production of PHB can be reduced by study of these two wastes in future.

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