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Economical production of poly-3-hydroxybutyrate by *Bacillus cereus*under submerged and solid state fermentation

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Abstract
High cost of poly-3-hydroxybutyrate production remained a major hindrance for its wide range applications. In the current study poly-3-hydroxybutyrate producing bacteria were isolated from environmental sources. Highest poly-3-hydroxybutyrate producing isolate *Bacillus cereus* PS 10 was investigated for its ability to use wide range of low-cost carbon sources including agro-industrial residues, viz. wood waste, potato peel powder, saw dust, maize bran, rice husk, molasses, whey etc. for poly-3-hydroxybutyrate production under submerged fermentation.*B.cereus* PS 10 exhibited remarkable metabolic capability and utilized most of the crude materials as carbon source for growth and poly-3-hydroxybutyrate production. Maximum poly-3-hydroxybutyrate yield was observed when glycerol (8.9 ±0.3g/L) and molasses (8.6 ±0.25 g/L) were used as carbon sources.Execution of solid-state fermentation (SSF) using malt as SSF substrate showed that *B. cereus* PS 10 grew successfully under SSF and produced appreciable poly-3-hydroxybutyrate yield (14.4 mg/g). Ability of *B. cereus* PS 10to utilize vast range of crude carbon sources for growth and poly-3-hydroxybutyrate production, and its capacity to grow and produce substantial poly-3-hydroxybutyrate yield under SSF reflects its potential connotation for industrial biotechnology.

Key words: poly-3-hydroxybutyrate), *Bacillus cereus*, submerged fermentation, solid state fermentation, agroindustrial wastes

1. Introduction
Mammoth disposal of non-degradable plastic wastes coupled with rapid diminution of fossil fuel feedstocks, and associated green house gas emissions has resulted in huge ecological sufferings. This motivated extensive research towards usage of renewable raw materials for production of alternatives of petroleum based plastics which are fully degradable [1-2]. Polyhydroxyalkanoates (PHAs) have attracted immense attention as suitable substitutes of petrochemical-based plastics because of their closely related material properties with that of various thermoplastics [3], and desirable characteristics like high molecular weight, biodegradability, and biocompatibility [4]. Polyhydroxyalkanoates are aliphatic bacterial polyesters accumulated intracellularly by many bacteria as carbon and energy sources under adverse conditions [5-6].Poly-3-hydroxybutyrate is one of the most widespread polyhydroxyalkanoate in nature, and represents a strong contender for bioplastic production. Poly-3-hydroxybutyrate is a non-toxic, biocompatible, biodegradable, and recyclable thermoplastic [3]. In addition, poly-3-hydroxybutyrate due to its distinctive properties like insolubility in water, resistance to hydrolytic degradation, impermeability to oxygen, resistance to UV, among others, may have potential for applications in biomedicine (tissue engineering), industrial and agricultural fields [7-8].

High yielding bacterial strains must be available should the poly-3-hydroxybutyrate production be economically feasible. Several Gram-negative and Gram-positive bacteria have been reported to accumulate poly-3-hydroxybutyrate intracellularly under defined environmental situations [9-12]. However, relatively fewer studies have been done on poly-3-hydroxybutyrate production from Gram-positive bacteria [3, 7, 12]. Poly-3-hydroxybutyrate from Gram-positive bacteria is free of endotoxins unlike that from Gram-negative bacteria

[13], and may have potential for application in biomedicine. Among several Gram-positive bacteria reported for poly-3-hydroxybutyrate production viz. *Clostridium*, *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces* and *Staphylococcus* [14], *Bacillus* spp. have gathered special attention due to several advantages like their fast growth and fermentation rates, ability to utilize wide range of complex agro-industrial wastes as substrates [3, 15], and easy amenability to genetic manipulations [16]. However, there are scanty reports of poly-3-hydroxybutyrate production from *Bacillus* spp. [6-7, 12, 15].

Despite appropriateness of poly-3-hydroxybutyrate as a potential alternative for petrochemical derived plastics its wide range industrial applications are hindered due to its high production cost. Carbon source alone may account for 50% of the total production cost [3]. Attempts have been made to utilize inexpensive agricultural/industrial residues as carbon sources viz. tapioca industry waste, palm jaggery, and horse gram flour [6], molasses [2], wheat bran, cassava powder, rice husk, ragi husk, jackfruit seed powder, corn flour [17-18], vinasse [9], sugar beet juice[19], crude glycerol from biodiesel industry [4, 20], among several others [7, 21-22], to substantially reduce the cost of poly-3-hydroxybutyrate production,

Poly-3-hydroxybutyrate production mostly has been accomplished using typical submerged fermentation, and rarely solid state fermentation (SSF) has been attempted [6, 21-22]. SSF is considered as an energy and cost saving process, and offers several advantages over submerged fermentation such as high efficiency, lower capital and operational costs, lower energy needs, among others [23]. Furthermore, abundantly available agricultural residues can be employed as solid substrates for production of various biotechnology-based products of industrial importance including poly-3-hydroxybutyrate [6, 23]. The major complexity related with SSF for poly-3-hydroxybutyrate production is the localization of the product. Poly-3-hydroxybutyrate being an intracellular product requires cells to be recovered from the SSF medium for poly-3-hydroxybutyrate extraction [6, 21].Poly-3-hydroxybutyrate production was attempted by *Ralstonia eutropha* in a SSF system using soy cake as solid substrate [21]. Tapioca industry waste was used as solid substrate for cultivation of *Bacillus megaterium* MSBN04 for poly-3-hydroxybutyrate production under SSF[6]. Polyurethane foam based inert support was developed for solid state culture of *Bacillus sphaericus* NII 0838 for production of poly-3-hydroxybutyrate [22].

Considering want for environment-friendly and economic approaches for poly-3-hydroxybutyrate production for its potential commercialization,the present study reports poly-3-hydroxybutyrate production from a recently isolated bacterium *Bacillus cereus* PS 10 [12] from municipal solid waste [24] using a range of low cost carbon sources including agricultural residues [25]. Also poly-3-hydroxybutyrate producing potential of *B. cereus* PS 10 was investigated under solid state fermentation using malt as solid substrate.

2. Materials and methods

2.1. Submerged fermentation for poly-3-hydroxybutyrateproduction

Bacteria isolated from natural sources viz. domestic waste landfills [24], agriculture field soil, compost, soil from automobile washing area, garden soil etc., were earmarked for their poly-3-hydroxybutyrate producing potential on the basis of plate assay and confocal microscopic analysisas described previously [12]. Selected bacteria were subjected to submerged fermentation in poly-3-hydroxybutyrate production medium (PHB-PM) which consisted of (g/L): glucose 20.0, malt extract 0.5, yeast extract 1.0, $MgSO_4.7H_2O$ 0.1, K_2HPO_4 0.5, and NH_4NO_2 0.1, at pH 7, for poly-3-hydroxybutyrate production. The bacterial culture was activated on nutrient agar slants for 24 h, and then inoculated in PHB-PM and grown at 30^{o}C under shaking (Innova, New Brunswick, USA) for 18 h to attain approximately 10^8 cfu/ml (Absorbance A_{600} 0.8-0.9) and inoculated (at 5%, v/v) into PHB-PM. Submerged fermentation was executed at 30^{o}C under shaking at 150 rpm. Flasks were removed at different time intervals (24-96 h) for extraction and quantification of poly-3-hydroxybutyrate.

2.2. Poly-3-hydroxybutyrateproduction using various carbon sources under submerged fermentation

A total of 26 carbon sources were examined for poly-3-hydroxybutyrate production using *Bacillus cereus* PS 10. viz., rice husk, wheat bran, malt, molasses, whey, wood waste, sesame oil cake, cotton cake, potato peel powder, walnut shell powder, malt spent wash, almond shell powder, wheat waste, maize bran, glycerol, lauric acid,

citric acid, acetone, castor oil, starch, mannitol, and butyric acid at 20 g/L. The effect of carbon source on poly-3-hydroxybutyrate production was determined by replacing glucose of the PHB-PM with either of the carbon source, and executing fermentation under shaking (150 rpm) at 30^{o}C.

2.3. Solidstate fermentation for poly-3-hydroxybutyrateproduction

Solid state fermentation (SSF) for poly-3-hydroxybutyrate production was performed [21] using malt as solid substrate. Malt was procured from local brewery 'Modern Divan Breweries', Jammu, India. Mineral salt solution (MSS) used as a moistening agent for solid substrate consisted of (g/L): magnesium sulfate 1, monopotassium phosphate 5, sodium chloride 0.1, ammonium nitrate 10, manganese sulfate 0.1, and calcium chloride 0.1 [23]. SSF medium consisted of a 5 g of malt and 10 ml of MSS. The malt (5 g) in 100 ml flasks was moistened with sterile MSS and contents were mixed well, and inoculated with freshly grown *Bacillus cereus* PS 10 biomass (at 15 mg). The flasks were incubated under static conditions at 30^{o}C for 24-96 h. After regular time intervals flasks were withdrawn, and contents were extracted with sterile phosphate buffer saline (50mM, pH 7.0) by applying vigorous agitation to remove the cells from solid substrate. The contents were filtered through Whatman filter paper, and the solid residue left was again extracted by vigorous agitation and filtered, and this was repeated 4 times to ensure complete recovery of cells from solid substrate. Filtrate obtained from all 4 steps was pooled, centrifuged and cell biomass obtained was treated with sodium hypochlorite solution for poly-3-hydroxybutyrateextraction, and poly-3-hydroxybutyrateestimation was done by concentrated sulphuric acid [12, 26].

2.4. Extraction and quantification of poly-3-hydroxybutyrate

Poly-3-hydroxybutyrate extraction from bacterial cells was done with sodium hypochlorite digestion method [12, 26]. The fermented broth of different time intervals or extracted biomass (cells) from solid substrate was subjected to centrifugation at 10,000 ×g for 15 min (Eppendorf centrifuge 5804-R, Germany) and the resultant biomass was used for poly-3-hydroxybutyrate extraction. The cell biomass was suspended in sodium hypochlorite solution and incubated at 37^{o}C for 1 h for complete digestion of cell components except poly-3-hydroxybutyrate. Samples were centrifuged at 8,000 ×g for 20 min to collect poly-3-hydroxybutyrate granules. The precipitated poly-3-hydroxybutyrate was washed with acetone and water, and dissolved in chloroform. Chloroform was allowed to evaporate overnight and the dried pellet obtained was used for poly-3-hydroxybutyrate estimation. The poly-3-hydroxybutyrate granules were mixed with 5 ml of concentrated sulphuric acid in capped glass tubes and heated for 10 min at 100°C in a water bath to convert the poly-3-hydroxybutyrate to crotonic acid. The samples were examined spectrophotometrically (UV1800 Shimadzu, Japan) at 235 nm against sulphuric acid blank. The poly-3-hydroxybutyrate concentration in different samples was determined from a standard curve prepared using standard poly-3-hydroxybutyrate (Sigma) [26].

3. Results and discussion

3.1. Poly-3-hydroxybutyrateproduction from various bacterial isolates under submerged fermentation

Preliminary screening for poly-3-hydroxybutyrate producing ability of bacterial isolates was done by plate assay and confocal microscopic analysis (Figure 1).

All the isolates which showed poly-3-hydroxybutyrate producing potential were subjected to submerged fermentation in the PHB-PM for production of poly-3-hydroxybutyrate up to 96 h. Time profile for poly-3-hydroxybutyrate production is a very significant parameter to be studied to know the exact fermentation time period required by the bacterial culture for producing maximum product yield. Results showed that bacterial isolate PS 10, isolated from domestic-waste landfills was the most potent poly-3-hydroxybutyrate producer, and identified as *Bacillus cereus* based upon biochemical and physiological examination, and 16S rDNA sequence analysis (sequence submitted to Genbank under accession no. *KF499032.1*), and designated as *B. cereus* PS 10 [12]. *B. cereus* PS 10 accumulated poly-3-hydroxybutyrate yield to the extent of 8.6 g/L ±0.25 after 48 h of fermentation. However, some other bacterial isolates viz., PS 16, PS 17, PS 22, PS 27, PS 28 also showed considerable amount of poly-3-hydroxybutyrate accumulation i.e. 3.3 g/L ±0.25, 2.8 g/L ±0.21, 3.3 g/L ±0.09, 3

g/L ±0.08, 3.1 g/L ±0.25, respectively. While, other isolates yielded comparably less amount of poly-3-hydroxybutyrate (Table 1).

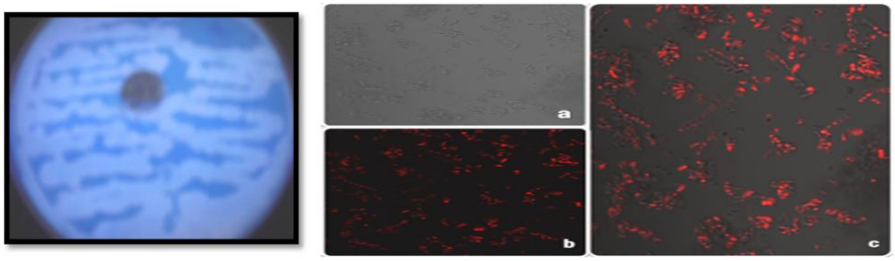

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Figure 1: Screening of bacterial isolates for poly-3-hydroxybutyrate producing ability based on plate assay (I), and confocal microscopic analysis (II):poly-3-hydroxybutyrate granules in bacterial cells in phase-contrast mode (a), in fluorescent-mode (b), and a combination of phase-contrast and fluorescent modes (c), at 60X magnification.

Table 1: Poly-3-hydroxybutyrate production by various bacterial isolates under submerged fermentation. Bacterial isolates were grown to required cell number (10^8 cfu/ml), and inoculated (at 5%, v/v) into poly-3-hydroxybutyrate production medium, and submerged fermentation was conducted under shaking (150 rpm) at 30°C.

Bacterial isolates	Poly-3-hydroxybutyrate concentration (g/L), Time (h) 24	48	72	96
PS 1	2.9 ±0.23	3.3 ±0.06	3.5 ±0.1	0.2 ±0.35
PS 3	0.5 ±0.2	3.05 ±0.12	0.4 ±0.09	0
PS 8	1.2 ±0.27	2.4 ±0.34	0.3 ±0.29	0
PS 9	1.9 ±0.03	2 ±0.14	1.2 ±0.21	0.02 ±0.05
PS 10	3.5 ±0.3	8.6 ±0.25	2.4 ±0.3	0.8 ±0.2
PS 11	2.5 ±0.08	2.9 ±0.12	0.8 ±0.21	0.1 ±0.05
PS 12	2.6 ±0.23	1.4 ±0.3	0	0
PS 14	2.7 ±0.22	2.9 ±0.25	1.4 ±0.16	0.2 ±0.14
PS 16	3.3 ±0.25	2.5 ±0.13	1.6 ±0.2	0.2 ±0.17
PS 17	2.7 ±0.15	2.8 ±0.21	1.0 ±0.09	0.04 ±0.18
PS 21	2.3 ±0.14	2.5 ±0.02	1.1 ±0.08	0.03 ±0.1
PS 22	0.2 ±0.13	3.3 ±0.09	2.9 ±0.16	0.4 ±0.02
PS 27	2.5 ±0.27	3 ±0.08	2.9 ±0.19	0.3 ±0.2
PS 28	3.1 ±0.25	2.7 ±0.07	1.6 ±0.3	0.7 ±0.05

Time-course (24-96 h) analysis for poly-3-hydroxybutyrate production from isolate PS 10 showed that poly-3-hydroxybutyrate synthesis started during first 24 h fermentation (Figure 2) and its accumulation increased significantly during the exponential phase and reached maximum at 48 h (8.6 ±0.25 g/L). However, after 48 h of fermentation poly-3-hydroxybutyrate production by isolate PS 10 got declined at 72 h (2.4 ±0.3 g/L)and reached lowest at 96 h (0.8 ±0.2 g/L).

Majority of the bacterial isolates exhibited highest poly-3-hydroxybutyrate yield after 48-72 h of fermentation (Table 1). After 72 h, minor decrease in poly-3-hydroxybutyrate content may be due to its utilization as carbon and energy source by bacterial cells. Optimum fermentation time for maximum poly-3-hydroxybutyrate production varies among different bacteria and depends mostly upon cultural/environmental conditions used during fermentation and genetic make-up of the organism [12].

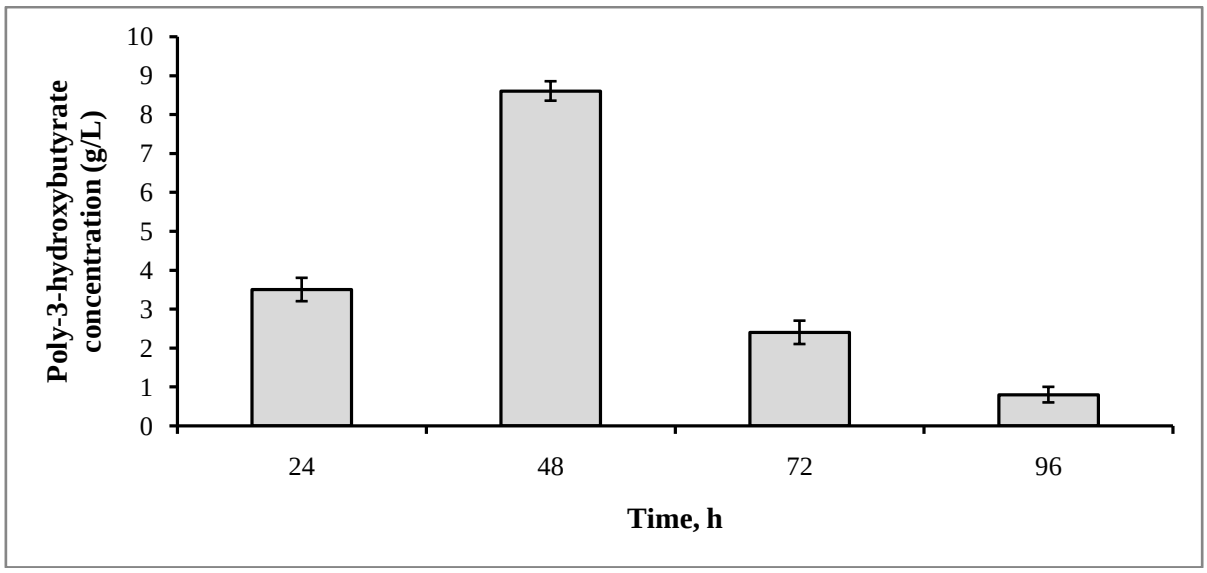

Figure 2: Time-course analysis for poly-3-hydroxybutyrate production by *Bacillus cereus*PS 10. *Bacillus cereus* PS 10 was grown for 18 h to attain required cell number (10^8 cfu/ml), and inoculated (at 5%, v/v) into poly-3-hydroxybutyrate production medium, and submerged fermentation was conducted under shaking (150 rpm) at 30^{o}C for different time intervals (24-96 h).

The poly-3-hydroxybutyrate production in *Bacillus subtilis* NG220 increased up to 72 h and afterwards, got declined probably due to utilization of poly-3-hydroxybutyrate by bacteria [15]. Highest polyhydroxyalkanoate production from *Bacillus cereus* strain 64-INS was reported after 21 h of fermentation [27]. *Alcaligenes* sp. accumulated maximum poly-3-hydroxybutyrate yield after 48 h of fermentation [2]. The poly-3-hydroxybutyrate accumulation by *Paenibacillus durus* BV-1 commenced at early stage of 16 h of incubation and continued during the log phase of growth, and sustained until late exponential phase [28]. Thus, time for maximum poly-3-hydroxybutyrate production depends on several factors like the type of microorganism, bioprocess conditions employed for cultivation and genetic potential of the microorganism.

3.2. Poly-3-hydroxybutyrateproduction using variouslow cost carbon sources

Considering carbon source being the major cost determining factor for poly-3-hydroxybutyrate production, usage of low cost agroindustrial wastes [25] has been widely practised for poly-3-hydroxybutyrate production [2-4, 9-12, 19-22]. However, poly-3-hydroxybutyrate yield may get affected due to use of various carbon sources in fermentation medium [12]. Poly-3-hydroxybutyrate synthesis occurs best in an environment with high carbon to low nutrient ratio. Considering the significant role of carbon sourcefor cell biomass synthesis, cell upholding and poly-3-hydroxybutyrate polymerization, it is utmost important to select an appropriate carbon source for bacterial fermentation for maximum poly-3-hydroxybutyrate production [28]. Sugars are the most regular substrates being used for poly-3-hydroxybutyrate production, however, lipid based substrates have also been exploited [4]. Results of the present study showed that *Bacillus cereus* PS 10 utilized wide range of carbon sources for growth and poly-3-hydroxybutyrate production which reflects the versatile enzymatic and metabolic potential of *B. cereus* PS 10 (Table 2).

Among the various carbon sources examined glycerol and molasses were earmarked as the most effective carbon sources for poly-3-hydroxybutyrate production from *B. cereus* PS 10, and supported a poly-3-hydroxybutyrate yield of 8.9±0.3 g/L after 72 h, and 8.6±0.25 g/L, after 48 h of fermentation, respectively. Poly-3-hydroxybutyrate yield on glycerol and molasses was comparable to that on glucose.

Table 2: Various carbon sources used (at 20g/L)for poly-3-hydroxybutyrate production by *B. cereus* PS 10 under submerged fermentation. Freshly grown bacterial cells (10^8 cfu/ml) were inoculated (at 5%, v/v) into poly-3-hydroxybutyrate production medium, and submerged fermentation was conducted.

Carbon source	Poly-3-hydroxybutyrate yield (g/L), Time (h) 24	48	72	96
Control (glucose)	3.5 ±0.3	8.6 ±0.25	2.4 ±0.3	0.8 ±0.2
Wheat bran	1.7 ±0.12	2.5 ±0.15	3.9 ±0.2	1.9 ±0.3
Malt	0.7 ±0.2	2.8 ±0.14	1.4 ±0.3	0.9 ±0.09
Lauric acid	0.3 ±0.07	1.5 ±0.18	2.4 ±0.3	0.4 ±0.02
Myristic acid	0.4 ±0.14	2.4 ±0.06	2.8 ±0.19	0.6 ±0.2
Whey	1.4 ±0.2	5.4 ±0.3	3.8 ±0.13	2 ±0.15
Glycerol	5.2 ±0.25	8.3 ±0.2	8.9 ±0.3	6.4 ±0.3
Potato peels	0.9 ±0.1	3.9 ±0.2	2.7 ±0.05	1.8 ±0.16
Rice husk	1.4 ±0.28	3.8 ±0.3	2.4 ±0.17	1.7 ±0.2
Wheat waste	0.6 ±0.14	2.7 ±0.2	1.7 ±0.1	0.9 ±0.03
Wood waste	0.3 ±0.08	0.8 ±0.06	0.6 ±0.02	0.5 ±0.14
Maize bran	3.7 ±0.2	5.3 ±0.15	4.4 ±0.3	2.2 ±0.1
Almond shell	3.2 ±0.2	6.6 ±0.25	3.8 ±0.17	1.4 ±0.09
Walnut shell	2.6 ±0.07	4.6 ±0.19	3.1 ±0.2	2.8 ±0.1
Cotton cake	0.3 ±0.08	2.6 ±0.1	2.4 ±0.13	1.2 ±0.2
Saw dust	0.2 ±0.07	1.3 ±0.2	0.9 ±0.1	0.4 ±0.02
Sesame cake	1.8±0.2	3.6 ±0.3	4.1 ±0.35	2.4 ±0.1
Molasses	3 ±0.2	8.6 ±0.25	5.2 ±0.2	3.3 ±0.25
Acetone	2.8 ±0.2	3.4 ±0.17	2.3 ±0.08	1.4 ±0.1
Castor oil	0.7 ±0.04	2.8 ±0.15	1.2 ±0.2	0.4 ±0.02
Starch	1.7 ±0.18	3.7 ±0.2	2.5 ±0.25	0.8 ±0.1
Citric acid	0.3 ±0.09	1.2 ±0.1	0.8 ±0.03	0.7 ±0.06
Mannitol	0.3 ±0.09	0.9 ±0.1	0.4 ±0.07	0.2 ±0.03
Malt spent wash	0.8 ±0.1	2.3 ±0.2	1.3 ±0.25	0.9 ±0.08
Ethanol	0.7 ±0.04	2.7 ±0.2	3.6 ±0.31	1.8 ±0.15
Butyric acid	0.9 ±0.07	1.2 ±0.12	1 ±0.1	0.9±0.04

Other carbon sources which supported considerable poly-3-hydroxybutyrate yield were acetone (3.4 ±0.17), ethanol (3.6 ±0.31), starch (3.7 ±0.2), rice husk (3.8 ±0.3), potato peels powder (3.9 ±0.2), wheat bran (3.9 ±0.2), sesame cake (4.1 ±0.35), walnut shell powder (4.6 ±0.19), maize bran (5.3 ±0.15), whey (5.4 ±0.3) and almond shell powder (6.6 ±0.25). However, carbon sources like wood waste, malt spent wash, sawdust, mannitol, citric acid and butyric acid could support a meagre poly-3-hydroxybutyrate yield (0.1-2.3 g/L ±0.02-0.28). Potential ability of *Bacillus cereus* PS 10 to utilize wide range of carbon sources for growth and poly-3-hydroxybutyrate production shows its enormous biochemical and metabolic capabilities which could be exploited for cost-effective production of poly-3-hydroxybutyrate using low-cost crude materials. Fermentation

time-based poly-3-hydroxybutyrate yield on molasses was 3±0.2 g/L (24 h), 8.6±0.25 g/L (48 h), 5.2±0.2 g/L (72 h) and 3.3±0.25 g/L (96 h), while that on glycerol it was 5.2±0.25 (24h), 8.3±0.2 (48 h), 8.9±0.3 (72 h) and 6.4±0.3(96 h) as presented in (Figure 3).

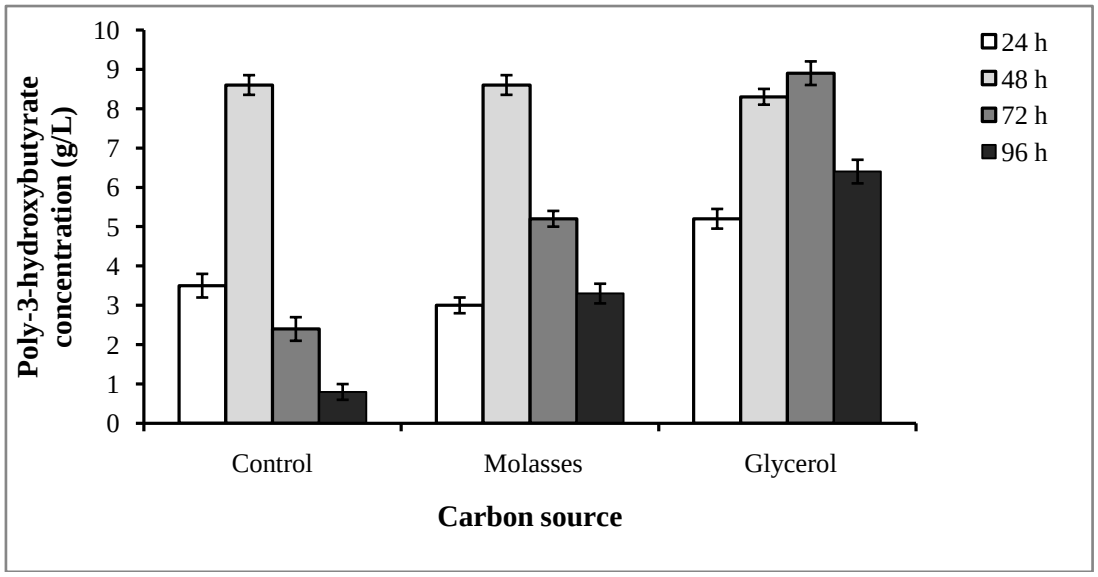

Figure3: Time-profile for poly-3-hydroxybutyrate production on molasses and glycerol. Glucose (control) of the poly-3-hydroxybutyrate production medium was replaced with molasses or glycerol (at 20g/L), and submerged fermentation was executed under shaking (150 rpm) at 30^{o}C for different time intervals (24-96 h).

Exploitation of low cost raw materials such as agroresidues and industrial wastes as substrates may not only contribute significantly towards curtailing cost of poly-3-hydroxybutyrate production but might have environmental benefits by addressing the disposal problems of wastes [2-4, 9-12, 19-22]. Utilization of agro-industrial by products like cane molasses and urea as carbon and nitrogen sources, respectively, resulted in poly-3-hydroxybutyrate production of 8.8 g/L after 48 h of fermentation by *Alcaligenes* sp. under optimized conditions [2]. Crude glycerol, a by-product from biodiesel fuel industry was used as an inexpensive carbon source (at 1 %, v/v) for production of poly-3-hydroxybutyrate content of 0.6 g/L from a recombinant *Escherichia coli* [4]. Poly-3-hydroxybutyrate production by *Bacillus subtilis* NG220 was attempted by utilizing sugar industry waste water supplemented with maltose and ammonium sulphate to get a poly-3-hydroxybutyrate yield of 5.29g/L [15]. Sugarbeet juice medium supplemented with nutrients other than sugar was utilized efficiently by *Alcaligenes latus* to grow and produce poly-3-hydroxybutyrate concentration of 4.01 g/L [19]. *Cupriavidus necator* utilized wheat straw under simultaneous saccharification and fermentation, and separate hydrolysis and fermentation conditions to yield 10.0 g/L and 7.1 g/L of poly-3-hydroxybutyrate, respectively [29]. Seven-fold enhanced poly-3-hydroxybutyrate production (27.3 g/L at 60 h of fermentation) was reported from a mutant of *Azotobacter vinelandii*that had altered poly-3-hydroxybutyrate regulation using a fed-batch fermentation process [30].

Several species of *Bacillus* have been reported to be good producers of poly-3-hydroxybutyrate [31]. *Bacillus firmus* NII 0830 utilized pentose sugar rich acid pretreated rice straw hydrolysate as a carbon source and produced poly-3-hydroxybutyrate to the extent of 1.69 g/L [3]. Rice straw hydrolysate produced by biphasic sulphuric acid pretreatment was reported to be an efficient substrate for poly-3-hydroxybutyrate production by *B. cereus* PS 10 [32]. Potato starch without any enzymatic or chemical treatment was found to be the effective carbon source for poly-3-hydroxybutyrate production from *Bacillus cereus* 64INS strain [27].A model-based control scheme was executed for improved polyhydroxyalkanoate production from organic wastes in a mixed culture two-stage system fed with synthetic wastewater [33]. Utilisation of agro-industrial residues for production of poly-3-hydroxybutyrate not only ensures the low production cost but also resolves the problem of

material waste management to a certain level. *Methylocystis* sp. WRRC1 produced a wide range of polyhydroxybutyrate-co-hydroxyvalerate copolymers (PHB-co-HV) when co-fed with methane and valerate or n-pentanol [34]. The ratio of HB to HV monomer was related to the concentration of valeric acid in the media. Copper-free growth conditions enhanced incorporation of HV and total polymer yield [34].

3.3. Solid state fermentation (SSF) for poly-3-hydroxybutyrateproduction using Bacillus cereus PS 10

SSF is a process in which microorganism is cultivated on moistened solid medium without the presence of free liquid phase. SSF has got several advantages over submerged fermentation including higher productivity, lower capital and operational costs, reduced generation of effluent etc. [23]. In the current study, malt was used as solid substrate for cultivation of *B. cereus* PS 10 for the production of poly-3-hydroxybutyrate. Malt is germinated barley grains which are dried, milled and used in breweries as basal substrate for beer production. Malt is nutritionally sufficient and contains various types of nutrients essential for growth of various microorganisms e.g. maltose 65%, complex carbohydrates30%, protein 3%, and several minerals [35]. It was observed that *B. cereus* PS 10 was capable of growing well on malt under SSF. Since poly-3-hydroxybutyrate is accumulated as intracellular inclusion bodies within the cytoplasm of the microorganism, it is important to efficiently extract the bacterial cells from the solid medium. The solid medium was subjected to vigorous agitation repeatedly with sterile phosphate buffer saline to ensure complete recovery of bacterial cells from the solid medium. Cell biomass was used for extraction and quantification of poly-3-hydroxybutyrate. It was observed that maximum poly-3-hydroxybutyrate yield of 14.4 mg/g of malt was obtained after 48 h of SSF (Figure 4).

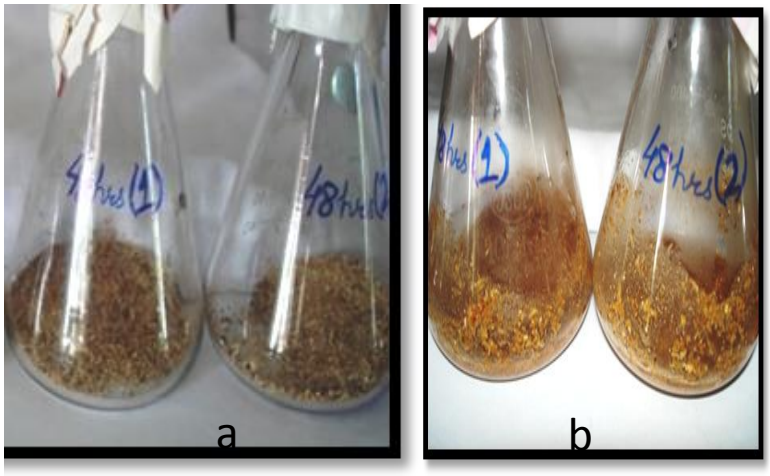

Figure4: Solid state fermentation for poly-3-hydroxybutyrate production from *Bacillus cereus* PS 10 using malt as solid substrate, ‘a’ and ‘b’ show carbon source prior to and after SSF, respectively. Five g malt was moistened with sterile mineral salt solution (10 ml) and inoculated with freshly grown *B. cereus*PS 10 biomass (at 15 mg).

Very few reports are available on poly-3-hydroxybutyrate production under SSF. Since poly-3-hydroxybutyrate is an intracellular product the key inbuilt difficulty associated with SSF is the retrieval of bacterial cells from solid substrate [6, 21]. However, SSF in addition to several merits provides possibility of utilizing agroindustrial wastes as raw materials, thus contributing to minimizing environmental problems and to reduce production costs. SSF has been successfully exploited for production of various products of industrial importance including poly-3-hydroxybutyrate [21-23]. A novel SSF bioprocess was developed in which polyurethane foam (PUF) was used as a physical inert support for cultivation of *Bacillus sphaericus* NII 0838 for poly-3-hydroxybutyrate production [22]. PUF may have several advantages as an inert support for SSF viz., it can soak up water and make it available to the microorganism for growth and metabolic activities, and it has high porosity, low density and comparatively high water assimilation capacity. SSF bioprocess was used an

economical technology for the production of poly-3-hydroxybutyrate from *Ralstonia eutropha* by employing soycake and babassu as solid substrates. Maximum poly-3-hydroxybutyrate yield of 1.2 mg/g and 0.7 mg/g was attained on soycake medium (after 36 h) and babassu cake medium after 84 h of fermentation, respectively [21]. Furthermore, addition of sugar cane molasses to soy cake enhanced poly-3-hydroxybutyrate production to 4.9 mg/g in 60 h. Optimization of SSF process was attempted for poly-3-hydroxybutyrate production from *Bacillus megaterium* MSBN04 using SSF-medium based on tapioca industry waste, palm jaggery, horse gram flour and trace element solution, and poly-3-hydroxybutyrate yield of 8.6 mg/g of tapioca industry waste substrate was obtained [6].

Appropriate moisture level, oxygen supply, mass transfer, heat transfer, diffusional limitations for substrates and metabolites, and heterogeneous conditions throughout the bioreactor i.e. formation of nutrient, pH, and temperature gradients, are some of the inherent challenges for SSF bioprocess [6, 21, 23]. Excess moisture results in decreased porosity of the substrate, changed particle composition of the substrate, can cause diffusional restrictions, and leads to reduced oxygen transfer, whereas low moisture content directs low solubility of the substrate nutrients, reduced bulge, and advanced water retention [21]. Agro-industrial residues are usually the preferred substrates for SSF and a number of such substrates viz., sugar cane bagasse, wheat bran, rice bran, maize bran, malt, gram bran, wheat straw, rice straw, rice husk, saw dust, corncobs, banana waste, tea waste, among several others have been used for cultivation of the microorganisms to produce desired products [23, 25, 36].

Conclusion

Current study concludes that *Bacillus cereus* PS 10 successfully utilized various low cost carbon sources for growth and production of poly-3-hydroxybutyrate. Glycerol and molasses supported maximum poly-3-hydroxybutyrate production under submerged fermentation. Furthermore, *Bacillus cereus* PS 10 performed well under solid state fermentation on malt and produced substantial amount of poly-3-hydroxybutyrate. Further study on bioprocess scale up must be executed to fully claim the potential of *B. cereus* PS 10 for poly-3-hydroxybutyrate production.

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