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Bacillus cereus Mediated Synthesis of 'Green Plastics'- Polyhydroxybutyrate

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Bacillus cereus **isolated from cloth used to smear oil on 'dosa' pan has been used for the production of PHB. The influence of medium components on the production of PHB was studied using Plackett Burman screening design which indicated that glucose concentration followed by yeast extract concentration play an important role in the production of PHB. The organism required glucose as carbon source at 0.5% and yeast extract as nitrogen source at 0.2% for the maximum production of PHB. The PHB production by *Bacillus cereus* increased from 34.9% to 52.4% (1.2 g of PHB/L) after optimization.**

Keywords: Polyhydroxybutyrate, *Bacillus cereus*, PBSD, Optimization.

The diminishing petroleum reserves combined with the negative impact of petroleum based plastics are forcing the scientific community to look for a potential alternative. Polyhydroxy alkanoates can be such an alternative as they possess properties similar to conventional plastics such as polypropylene making them applicable for a wide range of applications [1]. These biopolymers have the advantage of completely degrading to water, carbondioxide and methane by anaerobic microorganisms under various environmental conditions[2].

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoates (PHB) produced by number of bacteria as granules using fatty acids, sugars and other carbon sources[3]. PHB is water insoluble, resistant to UV radiation and impermeable to oxygen making them suitable for use as food packaging material[4]. The biodegradable and biocompatible nature of PHB makes it suitable for developing novel medical devices and tissue engineering[5, 6].

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These bacterial biopolymers are formed as an intracellular reserve material in response to imbalance in the growth environment where a suitable carbon source is present in excess and one or more nutrients are limiting, example, nitrogen, phosphorus, sodium, oxygen, magnesium, manganese[7,8]. The amount of PHB content produced is greatly influenced by the strain of microorganisms, the type of substrate used and its concentration[9,10]. Therefore, an attempt has been made in this study to identity the medium components that play an important role in the production of PHB by *Bacillus cereus* isolated from oil amended cloth used to smear oil on 'dosa' pan by Plackett Burman screening design followed by optimization by conventional method.

MATERIALS AND METHODS

The microbiological growth media and the chemicals used were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India and SISCO Research laboratory, Mumbai, India. Solvents used in the studies were of AR grade and were purchased from Merck Pvt. Ltd.

Microorganisms and Culture conditions

A bacterial culture showing appreciable PHB production was isolated from oil amended cloth (used to smear oil on 'Dosa' pan). The mineral salt medium used in the initial screening studies for PHB production was of the following composition (g /L) 2.5 K_2HPO_4, 2.5 KH_2PO_4, 5 glucose, 2 $(NH_4)_2HPO_4$, 0.2 $MgSO_4.7H_2O$, 0.01 $FeSO_4$. 7 H_20 and 0.007 $MnSO_4.7\ H_2O$. The pH of the medium was adjusted to 7.5. The medium without glucose was sterilized at 121^{0}C for 20 min. Glucose was sterilized separately and added to the medium. Wherever the effects of nitrogen sources were studied, $(NH_4)_2HPO_4$ in the medium was replaced with NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, $NaNO_3$, yeast extract, beef extract and peptone. The effect of carbon sources was studied by replacing glucose with lactose, sucrose, fructose and maltose.

PHB extraction and quantification

The bacterial cells after 48 h of incubation were harvested by centrifugation at 5,000 rpm for 10 min and were dried. The dried cells were treated with boiling chloroform to dissolve the PHB along with lipids. This solution was filtered using Whattman No.1 filter paper to remove the cell mass. The filtrate was treated with methanol which selectively precipitates PHB from the solution [11]. This solution was further subjected to centrifugation at 12,000 rpm for 10 min to precipitate the PHB and was air dried. The dried PHB was weighed and stored for further studies.

Plackett Burmann Screening Design for media components

Plackett Burman methodology (PB) was used to screen (n) variables in (n+1) number of experiments [12]. The PB matrix was developed manually and the screening design was set up for seven variables in two levels, high and low (Table 1). Dipotassium hydrogen phosphate and Potassium dihydrogen phosphate were dummy variables.

RESULTS AND DISCUSSION

The bacterium was identified based on their biochemical and molecular characterization as *Bacillus cereus* [13]. *Bacillus* sp. are well known for their ability to produce PHB. In one of the study, 29 *Bacillus* species have been isolated and screened for their ability to produce PHB in which Yilmaz et al.[14] have reported that *Bacillus brevis* followed by *Bacillus cereus* accumulated appreciable amount of PHB qualifying them as a potential candidate for industrial application.

Plackett Burman Screening Design

The experimental results show a wide variation in PHB production from 0.24 to 0. 82 g /L which reflected the importance of medium components for higher PHB production. Dipotassium hydrogen phosphate and Potassium dihydrogen phosphate were dummy variables. Table 2 and Table 3 show experimental results for PB screening design and associated significant levels respectively. Glucose concentration showed larger effect followed by yeast extract concentration. It can be seen with low concentration of glucose and yeast extract and high temperature, PHB production by the *Bacillus cereus* was predominating, provided the temperature and agitation are held high with pH maintained at 6.

Table 1. Variables and their levels selected for PBSD

	Variables	Units	Low (L)	High (H)
A	Glucose	g/100 ml	1	10
B	Yeast extract	g/100 ml	0.1	0.5
C	K_2HPO_4	g/100 ml	0.1	0.5
D	KH_2PO_4	g/100 ml	0.1	0.5
E	Temperature	°C	20	40
F	pH		6	9
G	Agitation	rpm	0	150

Table 2. PB design matrix with experimental values for PHB production by *Bacillus cereus*

Trials	A	B	C	D	E	F	G	PHB (g/L)
1	H	H	H	L	H	L	L	0.42
2	L	H	H	H	L	H	L	0.56
3	L	L	H	H	H	L	H	0.82
4	H	L	L	H	H	H	L	0.36
5	L	H	L	L	H	H	H	0.22
6	H	L	H	L	L	H	H	0.41
7	H	H	L	H	L	L	H	0.28
8	L	L	L	L	L	L	L	0.73

Optimization of Carbon source

Carbon source play an essential role in PHB production. Even though all the carbon sources tested except maltose supported appreciable biomass production, PHB production was maximum with glucose as carbon source followed by sucrose (Fig. 1a). In one of the studies the highest level of PHB accumulation was observed in the medium with glucose as carbon source in *B.subtilis* 25, *B. megaterium* 12 [15,16]**.** Wu et al. [17] have reported that *Bacillus* sp. JMa5 strain accumulated 25-35%, (w/w) PHB during sucrose fermentation. In another study, *A. eutrophus* efficiently produced the maximum concentration of PHB with glucose in comparison to other carbon sources [2]. *Pseudomonas aeruginosa* was reported to accumulate 60% PHA when sugarcane bagasse was used as carbon source [7]. Results of experiments conducted with different concentrations of glucose have shown 0.5% as an optimum concentration for maximum production of PHB (Fig. 1b). At lower glucose concentration

Table 3. PBSD results and significant levels

	A	B	C	D	E	F	G
EH	0.147	0.148	0.221	0.202	0.182	0.155	0.173
EL	0.233	0.232	0.159	0.178	0.198	0.225	0.207
D	-0.0215	-0.021	0.0155	0.006	-0.004	-0.0175	-0.0085
MS	0.000925	0.000882	0.000481	7.2E-05	3.2E-05	0.000613	0.000145
MSE				0.00027625			
F TEST	3.346606	3.19276	1.739367	0.260633	0.115837	2.217195	0.523077

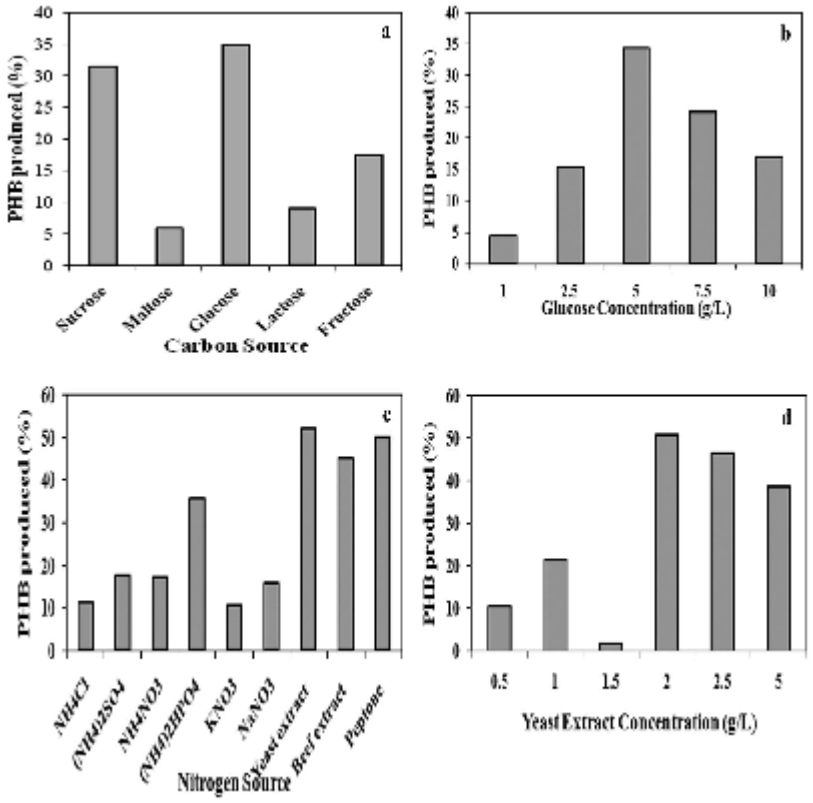

Fig. 1. Effect of different nutrient supplement on PHB production by *Bacillus cereus* (a) carbon sources (b) glucose concentration (c) nitrogen sources and (d) yeast extract concentration

the biomass production as well as the PHB production was low which may be explained based on the requirement of carbon source for the growth of the organism. At higher concentrations, even though the biomass production was more PHB production was less. This proves the fact that maximum PHB production takes place under stress conditions [18].

Optimization of Nitrogen source

Another important component that plays a vital role in polyhydroxyalkanoate production is the nitrogen source. PHB production was studied with different organic and inorganic nitrogen sources. All the inorganic nitrogen sources tested except diammonium orthophosphate gave considerably less biomass and PHB production whereas all the organic nitrogen sources gave maximum amount of Biomass and PHB production with yeast extract giving maximum biomass and PHB (Fig. 1c). Similar results were reported in which maximum biomass and biodegradable polymers were produced when yeast extract used as the nitrogen source by *Alcaligenes faecalis* [19]. In another study the highest level of PHB accumulation was observed in the media with proteaz peptone as nitrogen source in *B. subtilis* and in *B. megaterium* [15]. The amount of PHB produced increased as the concentration of yeast extract was increased up to 0.2% above which there was no appreciable change in the amount of PHB produced (Fig. 1d).

CONCLUSION

The microbial production of PHB is gaining importance owing to the benefits offered in terms of biocompatibility and biodegradability. In the present investigation, Plackett Burman screening design followed by conventional optimization resulted in the enhanced production of PHB by *Bacillus cereus*. Glucose at a concentration of 0.5% and Yeast extract at 0.2% were found to support maximum amount of PHB production. The future plan of work includes the use of agricultural waste like sugarcane bagasse and oil cakes for the production of PHB by *Bacillus cereus*.

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