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Production of Poly-3-hydroxybutyrate from Inexpensive Substrates

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***Bacillus megaterium* 79/A, a starch and lactose utilizing strain, produced 0.90 g/l PHB with corn flour as sole carbon substrate; this was followed by other flours like, soya bean, wheat etc. *Bacillus megaterium* 79/A produced 0.62 g/l PHB with treated whey of 46.2% concentration and 0.55 g/l PHB with the same concentration of untreated whey. When 5mM microcosmic salt was used as an additional supplement, the organism produced 0.95 g/l PHB and 0.80 g/l PHB with 46.2% treated whey and 46.2% untreated whey, respectively.**

Key words: *Bacillus megaterium*, Lactose, PHB, Starch, Whey.

Polyhydroxyalkanoic acids (PHA) (Poly-3-hydroxybutyrate (PHB), a representative compound of the family of PHA) are common cellular granules found in prokaryotes. In PHB production, about 50% of the total production cost is for raw material (Anderson and Dawes 1990). It has been studied that the cost of carbon source is critical for reducing the production cost of PHA (Akiyama *et al.,* 2003). The high production cost of PHB can be decreased by strain development, improving fermentation process, separation process and by using a cheap carbon source (Kim 2000). Thus, the use of an alternate cheap carbon source is required in order to reduce the high production cost of PHB.

One such cheap substrate is starch, a renewable carbon source, available abundantly from plant sources. Previously, use of starch is limited due to pre-treatment with either enzyme or acid. The need for identification and exploitation of a bacterial culture for the coproduction of starch hydrolyzing enzyme and PHA production has arrived recently (Halami 2008).

The second cheap substrate is whey, a major by-product of cheese or casein form bovine milk. It contains approximately 4.5% (w/v) lactose, 0.8% (w/v) protein, 1% (w/v) salt and 0.1 to 0.8% (w/v) lactic acid (Yang *et al.,* 1994). This material needs high amount of dissolved

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oxygen (DO) for its disposal, hence, it is regarded as a pollutant.

In this paper, starch and whey were tested for PHB production with the *Bacillus megaterium* 79/A, a starch and lactose utilizing strain. For this purpose, the plant starch materials like various pulse/cereal flours spilled on the floors of flour mills and whey, lactose containing dairy waste were used.

MATERIAL AND METHODS

Isolation and identification of *Bacillus megaterium* 79/A

Bacillus megaterium 79/A was isolated from soil sample and identified by its physiological and morphological characteristics (Priest and Alexender, 1988).

Production of PHB from starchy materials

For this purpose, flours from different pulses/cereals, such as, pigeon pea, red lentil, black gram, bengal gram, green gram, corn, soya bean, wheat, rice and jowar were collected from local flour mills and tested as substituted cheap carbon substrates.

These different substrates were used in different concentrations in the E2 media substituting the carbon substrate. The concentrations studied include, 5 – 25 g/l (w/v). The experiments were done with all other optimal conditions and in triplicates. The means of the results of experiments conducted in triplicates were presented in this paper.

Production of PHB from whey

Bacillus megaterium 79/A was grown in a medium containing whey as a source of carbon and nitrogen, since it is available as a cheap substrate for PHB production. E2 medium was modified for this purpose by substituting glucose with different concentrations of whey.

Whey obtained from local dairy (Vijaya Dairy, Hyderabad) was acidified to pH 4.5 with 1N HCl, heated to 74°C for 15 min, cooled and centrifuged at 10,000 g for 15 min at 4°C (Yellore and Desai 1998). The supernatant obtained after the above treatment (whey supernatant) and the whole whey (untreated whey) were used in the experiments after adjusting the pH to 7.0 with alkali. Concentrations of the treated and untreated whey in PHB production experiments include: 18.5 %, 27.5 %, 37.0 %, 46.2 %, and 55.5 %. Study of PHB production from whey in combination with the inorganic nitrogen source, microcosmic salt was also included. For this, different concentrations of microcosmic salt in the range of 5 mM to 15 mM were employed.

Analytical methods

After inoculation and incubation of *Bacillus megaterium* 79/A in medium, the following analysis was done. Each sample was used for the determination of the cell dry weight (CDW) and PHB content in the bacterial cells. The cell concentration was determined by measuring CDW, where 5 ml culture broth was centrifuged, pellet obtained was washed and dried at 105°C until the weight did not decrease further. PHB estimation was carried out according to UV spectrophotometer method (Aslim *et al.,* 1998). PHB (%) was defined as the percentage of the ratio of PHB to CDW.

RESULTS AND DISCUSSION

Production of PHB from starchy materials

On analysis of the economics of PHB production, the cost of the carbon substrate alone accounts for 50% of the total expenses of PHB production. In order to reduce down the cost of production, it is essential to identify the microorganisms that utilize cheap carbon sources efficiently to produce PHA (Kim 2000). Starch, available abundantly from plant sources has recently been used as a carbon source for PHB production. Use of starch in this process needs enzymatic hydrolysis (Huang *et al.,* 2006), acidogenesis of starchy waste water in a reactor to form volatile fatty acids (Yu 2001).

Bacillus megaterium 79/A, was used to investigate the use of waste starchy materials, such as, various cereal and pulse flours available in abundant as spills in flour mills for PHB production. The ability of this organism to hydrolyze starch due to its intrinsic amylase activity was considered and starch was used as the sole carbon source for growth and PHB production. Ten different pulse/cereal flours, in five different concentrations (5-25 g/l) were used in the investigation of PHB production by *Bacillus megaterium* 79/A (Table 1). The highest yield of PHB was found with corn flour as the starchy

material. Table 1 results showed that, *Bacillus megaterium* 79/A was able to accumulate a maximum of 0.90 g/l PHB within 34 h of incubation when corn flour was used as carbon substrate, suggesting a very faster rate of polymer synthesis, when compared to all other substrates. This was followed by other flours from black gram, wheat, red lentil, soya bean, jowar, rice, green gram, pigeon pea and bengal gram. Same concentration of PHB (0.88 g/l) was produced with wheat flour and black gram flour as carbon substrates at 25 g/l concentration in the modified E2 medium. All these cereal and pulse flours yielded PHB in the range of 0.90 g/l to 0.19 g/l. Though, the PHB concentration was less when compared to PHB concentration from optimal carbon and nitrogen substrates, this could be a significant investigation because as mentioned in many earlier reports, the cost of carbon substrate is very much important in the economical production of PHB from bacteria.

Production of PHB from whey

Whey, a lactose containing dairy industry waste, was used to produce PHB by *Bacillus megaterium* 79/A. The untreated whey at 46.2% concentration in the medium yielded, 0.55 g/l PHB, whereas, the treated whey at the same concentration in the medium yielded only 0.62 g/l PHB (Table 2). The addition of microcosmic salt at 5 mM concentration to the media with either treated or untreated whey at 46.2% concentration increased the amount of PHB production by the strain. A highest of 0.95 g/l PHB was produced by the *Bacillus megaterium* 79/A in medium with 46.2% treated whey and 5 mM microcosmic salt. Similar concentrations of untreated whey and microcosmic salt yielded 0.80 g/l PHB (Table 3).

Table 1. PHB produced (g/l) from various concentrations of flours

Conc of flour	Bengal gram	Pigeon pea	Soya bean	Red lentil	Corn	Wheat	Jowar	Rice	Black gram	Green gram
5 g/l	0.33	0.32	0.42	0.29	0.44	0.48	0.19	0.55	0.29	0.39
10 g/l	0.38	0.36	0.30	0.32	0.46	0.52	0.30	0.59	0.38	0.43
15 g/l	0.44	0.33	0.40	0.46	0.51	0.56	0.52	0.64	0.44	0.53
20 g/l	0.28	0.23	0.66	0.61	0.64	0.80	0.57	0.70	0.49	0.69
25 g/l	0.31	0.50	0.78	0.85	0.90	0.88	0.77	0.75	0.88	0.72

Table 2. PHB (g/l) produced from whey.

% of whey	18.5%	27.5%	37.0%	46.2%	55.5%
Treated whey	0.49	0.58	0.24	0.62	0.50
Untreated whey	0.41	0.41	0.57	0.55	0.33

Table 3. PHB (g/l) produced from 46.2% whey together with the different concentrations of microcosmic salt

Concentration of microcosmic salt (mM)	Treated whey + microcosmic salt	Untreated whey+ microcosmic salt
5.0	0.95	0.80
7.5	0.68	0.59
10	0.41	0.39
12.5	0.24	0.19
15	0.17	0.08

CONCLUSIONS

Many workers have explored various easily available agro-industrial wastes as carbon sources, such as, sugarcane molasses, date syrup and soy molasses for the production of PHB employing *Bacillus* spp. (Gouda *et al.,* 2001; Omar *et al.,* 2001; Full *et al.,* 2006).

The native *Bacillus megaterium* 79/A has desirable properties of tolerance to increased conditions of pH and temperature (data not shown). Such strains are found to be better suited for industrial production of PHB in order to minimize contamination. The nonpathogenic features of the *Bacillus* sp. ensure safety while handling. This strain of *Bacillus megaterium* showed great ability to ferment various carbohydrates by recycling agro-industrial wastes; hence can be effective in PHB production at industrial level.

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