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Microbial production of poly-3-hydroxybutyric acid from soybean oil by *Bacillus subtillis*

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ABSTRACT

Polyhydroxyalkanoates(PHAs) are biodegradable polymers produced by microbes to overcome environmental stress condition. Commercial production of PHAs due to its high cost is limited compared to that of conventional polymers. Another hindrance is the brittle nature and low strength of Poly-3-Hydroxybutyric Acid (PHB), the most widely studied PHA. The needs are to produce PHAs, which have better elastomeric properties suitable for biomedical applications, preferably from inexpensive renewable sources to reduce cost. Certain unique properties of Bacillus subtilissuch as lack of the toxic lipo-polysaccharides, expression of self-lysing genes on completion of PHA biosynthetic process – for easy and timely recovery, usage of bio wastes as feed enable it to compete as potential candidate for commercial production of PHB. Bacillus subtiliswas found to produce PHB and the amount of PHB produced was estimated under various conditions like pH, temperature, and also using different substrates. The amount of PHB produced was estimated by reading the absorbance at 235nm as well as data is studied by using statistical analysis. When relationship between oil consumption and cell growth were studied 5 g/L soybean oil was considered as suitable due to highest producuction of CDW and PHB content (13.1 g/L) and (87 wt%) respectively.

Keywords: Polyhydroxyalkanoates, Poly-3-Hydroxybutyric Acid, Soybean oil, Cell Dry Weight, UV Spectrophotometer, Tukey's HSD test

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INTRODUCTION

Humans in developed countries have grown accustomed to life in a “plastic society”. Most plastics end up in landfills on our shores or in the ocean. It is increasingly being realized that the use of long-lasting polymers for short-lived applications is not entirely justified, especially when increased concern exists about the preservation of finite resources [1].

Biodegradable plastic is rather new and promising because of its actual production and utilization by bacteria to form biopolymer. Bioplastics are biodegradable, compostable and bio erodible plastics. These polymers, usually lipid in nature, are accumulated as storage material [in the form of mobile, amorphous, liquid granules] in microorganisms and allow microbial survival under stress conditions. This storage material is known as polyhydoxyalkanoates(PHAs), which store carbon and energy, when nutrient supplies are imbalanced. These

polyesters, known as Bio plastics contain long chains of monomer which join with each other by ester bond [2]. Bacteria from the genera *Alcaligenes, Bacillus, Azospirillum, Pseudomonas etc.* produces polymers in the PHA family which are used for energy and as a storage form of cellular carbon [3]. These microorganisms can accumulate from 30-80% of their dry weight in PHA. The various inexpensive substrates that are used for the production of PHB include whey, starch containing substances like arrowroot, rice water and sago water [3]. PHA is biodegradable, water insoluble, non-toxic, bio-compatible, piezoelectric, thermoplastic, and/or elastomeric. These features make them suitable for applications in the packaging industry and as substitute for hydrocarbon-based plastics [4]. It has wide applications in different areas such as packaging material, long term dosage of drugs, medicines, insecticides, herbicides, fertilizers, cosmetics, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, cups etc. Several studies are in progress for its relevance in medical field for bone replacements and plates, surgical pins, sutures, wound dressings, and blood vessel replacements [5].

So this study aimed to focus on production of PHB as they are the promising technology that can change the scenario of plastic waste management.

MATERIALS AND METHODS

BACTERIAL STRAIN AND MEDIA

Bacillus subtillis was used in this study isolated from edible oil contaminated soil samples which were collected from various oil industries through north Gujarat region and cultivated in nutrient rich [NR] medium containing 2 g/L yeast extract, 10 g/L meat extract and 10 g/L peptone . Small amounts of Soybean oil [final concentration 1 g/L] were added to the NR medium, to study the effects of adding the oil to the preculture. Preculture without oil addition was used as control. The bacterial cells were maintained on agar mineral salts medium [MSM][6] supplemented with 2% [w/v] of fructose.

CARBON SOURCE

Soybean oil purchased from local market of Patan[Gujarat] was used as the sole carbon source for PHB biosynthesis. The oil was filtered and autoclaved separately before it was added to MM for PHB biosynthesis [7].

FERMENTATION AND PRODUCTION OF PHB

The production of PHB or copolymer was carried out by two-stage cultivation [8]. The isolated organisms were cultivated in the nutrient broth medium without any nutrient limitation, at 37 °C and 200 rpm for 24 hrs.

After incubation, 2 ml of culture was taken to inoculate the flask containing 200 ml of sterile production medium, and all the isolates were first grown for 72 hrs. at 37 ^{o}C with shaking at 200 rpm in a carbon – rich MSM containing sesame oil [1% w/v] as a sole carbon source.

To study dry cell weight [DCW] of bacterial cell and determine effect of various optimizing parameters, cells accumulating PHB were cultivated in 250 ml of modified mineral salts basal medium [MMSB][7] which contains $[NH_4]_2HPO_4$ [1.1g], K_2HPO_4 [3.7g], 10 ml of 0.1M $MgSO_4$ and 1.0 ml of a microelement solution [this microelement solution contained $FeSO_4.7H_2O$ [2.78 g], $MnCl_2.4H_2O$ [1.98 g], $CoSO_4.7H_2O$ [2.81 g], $CaCl_2.2H_2O$ [1.67 g], $CuCl_2.2H_2O$ [0.17 g], and $ZnSO_4$-$7H_2O$ [0.29 g] in 1 l of 1N HCl]. The pH was adjusted to 7.00 and then four edible oils 1% [w/v] each were taken separately in different 500 ml Erlenmeyer flasks and incubated for 72 hrs. on rotary shaker at 250rpm, at 37 ^{o}C . At the end of the fermentation, the cells were harvested by centrifugation [6000 g, 10 min, 4^{o}C]. Cell pellets were washed with hexane to remove residual oils and then with distilled water. The washed cell pellets were frozen at 20 ^{o}C for 24 h prior to storage in liquid glycerol [Marjadi and Dharaiya, 2012]. The organisms were confirmed for presence of PHB granules and lipase productions were confirmed as described by [9].

Lipase activity assay

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long- chain fatty acids [10]. Lipase activity was measured spectrophotometrically[11] with some modifications by using p-nitrophenyllaurate[pNPL] as the substrate. The emulsion solution was prepared by dissolving 5 mM of pNPL in 10 mL of dimethyl sulphoxide[DMSO], which was then emulsified in 90 mL of phosphate buffer [100 mM, pH 7] containing 0.1% [w/v] polyvinyl alcohol [PVA] and 0.4% [w/v] Triton X-100.

Approximately 100 mL of cell-free supernatant samples were mixed with 1.9 mL of the emulsion and incubated for 10 min at 30 ^{o} C. The absorbance was measured at OD 410 by spectrophotometer. One enzyme unit was defined as the amount of enzyme that liberates 1 mmol of p-nitrophenol per min under the incubation condition.

RESIDUAL OIL ANALYSIS

Residual oil analysis was carried out by mixing 1 mL of cell-free supernatant in 5 mL of hexane [Loba®]. As described in Figure-1 fermented broth was treated with hexane and water to remove oil. The mixture was vortexed for 1 min to ensure that all the residual oil in the supernatant had completely dissolved. After that, the mixture was allowed to settle until two distinct immiscible layers were observed [12].

Approximately 1 mL of the hexane layer [top] consisting of oil was transferred to a pre weighed plastic plate and left to dry in a fume hood until a constant weight was obtained. The oil remaining on the plate was weighed.

CELL DRY WEIGHT [CDW] MEASUREMENT

Cell dry weight was determined at the end of the cultivation [72 hrs.]. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes.

The cell pellet was then re suspended in 100 ml of hexane by vortexing and then centrifuged again at 10,000 rpm for 5 minutes to remove remaining oil.

Here, 10 ml of culture sample was centrifuged at 13,000 rpm for 15 minutes at 4^{o}C. The pellet was resuspended in distilled water [10ml] and centrifuged again for washing. . The washed cells were dried at 105 ^{o}C for 24 hrs. in a hot air oven and then allowed to cool down. The drying was repeated until constant weight was obtained
[13]. Cell concentration was expressed as dry cell weight [DCW][g/l].

EXTRACTION OF AND ESTIMATION OF PHB

5 ml of culture was centrifuged at 10, 000 g for 10 minutes and supernatant was discarded. The pellets were suspended in 2.5 ml of 4 % sodium hypochlorite for digestion and 2.5 ml of hot chloroform and were incubated at 37°C for 1 hour. The extractions of native PHB granules were performed as described by [9]. The amount of PHB in the extracted samples was determined spectrophotomertrically at 235 nm [14, 15].

OPTIMIZATION OF PHB PRODUCTION USING DIFFERENT CONCENTRATION OF SOYBEAN EDIBLE OIL AS CARBON SOURCE: Exactly how different concentration of soybean oil affected PHB production was evaluated by providing 0.5 to 10.5 g/L ml of Soybean oil in different flask containing same MMSB medium and were tested for the better productivity of PHB. All the selected bacterial cultures were inoculated and incubated at 30 °C for 72 hrs. After 72 hrs.of fermentation, PHB produced were extracted and measured with spectrophotometer at 235 nm [14,15].

RESULTS AND DISCUSSION

Lipid inclusion granules were stained black whereas the bacterial cytoplasm was stained pink in color which confirmed the presence of Lipid inclusion granules inside the bacterial cell [9].

As crotonic acid possess α, β, unsaturated bond and has an absorption maximum at 235 nm and hence the amount of PHB in the extracted samples was determined with UV spectrophotometer at 235 nm with reference to the standard graph of 3- hydroxy butyric acid.

Mean data accompanied by different letters are significantly different [Tukey's HSD test, $p < 0.05$].

Effects of Soybean oil concentration on PHB biosynthesis

Preliminary studies exhibited that soybean oil purchased from local market of Patan, Gujarat, India showed PHB accumulation and CDW increased while no residual oil was observed. These results indicated that the oil was consumed completely by the bacterial cells for their growth and simultaneously produced PHB. The PHB accumulation and CDW increased while no residual oil was observed as soybean oil concentration increased from 0.5 to 10 g/L [Fig. 2]. These results indicate the consumption of oil for cell growth and PHB production. The CDW [13.1 g/L] and PHB content [87 wt%] were found highest when 5 g/L soybean oil was used. However, the CDW and total PHB accumulation decreased while the residual oil increased after the 5 g/L concentration.

Effects of adding oil in preculture medium

To study the effects of soybean oil addition to the preculture medium, 1 g/L of soybean oil was added to the MSM preculture medium during inoculum preparation.

The lipase activity increased up to 88% in the initial stage [0-18 h] of cultivation when 1 g/L of oil was added to the preculture medium [Fig. 3]. After 18 h, the lipase activity [13 U/mL] in samples with oil in preculture plateaued and then dropped steadily after 72 hrs. When no oil was added to the preculture medium, the lipase activity could be graphed as a bell-shaped curve but shifting to the right of the fermentation timeline with the lipase activity plateau at 24 hrs. [11 U/mL] and starts to drop after 72 hrs.reflecting that adding oil into the pre culture medium may induce the production of lipase in the initial stage of fermentation.

Figure- 4 clearly depicts that pre culture with and without oil resulted in similar cell growth and PHB accumulation and that both treatments peaked at the 72 hrs. mark and then remained steady with no significant differences beyond 120 h. The final cell growth and PHB content were not significantly affected by the addition of 1 g/L of soybean oil to the preculture.

Most of the plastics and synthetic polymers are produced from petrochemicals. Because of their persistence in the environment, several communities are more sensitive to the impact of discarded plastics on the environment. Consequently, for the past two decades, there have been growing public and scientific interests in the development and use of biodegradable polymers as an ecologically useful alternative to plastics [16].Natural polymers are synthesized and accumulated by a diverse group of microorganisms [17].

Hydroalkanoic acids have been detected in some microorganisms and if they are exploited for optimized PHB production this may reduce production cost and help in large scale uses. The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics [18].

According to National Council of Applied Economic Research [19] in India, different varieties of edible oil are consumed, generally depending on the regional preferences and availability. India is one of the largest producers and consumers of edible oil in the world. Indian Vegetable Oil Industry comprises around 1,50,000 oilseed crushing units with a total capacity of 425 million tones, 800 solvent extraction units with capacity of 345 million tons of the oil-bearing material, about 300 refineries with capacity of 50 million tones and 205 vanaspati units with annual capacity of 32 million tones.

The soybean is economically the most important bean in the world, providing vegetable protein for millions of people and ingredients for hundreds of chemical products [20]. Soybean [Glycine max] also known as Golden Bean is the largest oilseed crop in world accounting for more than 50% of the world oilseeds production. Above 80% of the global soybean output is crushed worldwide to obtain oil and meal[21].

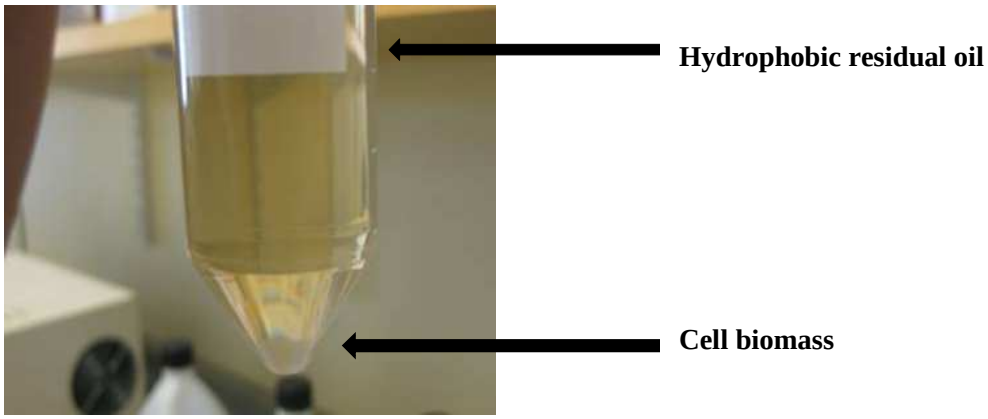

Figure 1 Fermented broth treated withhexane and water to remove oil

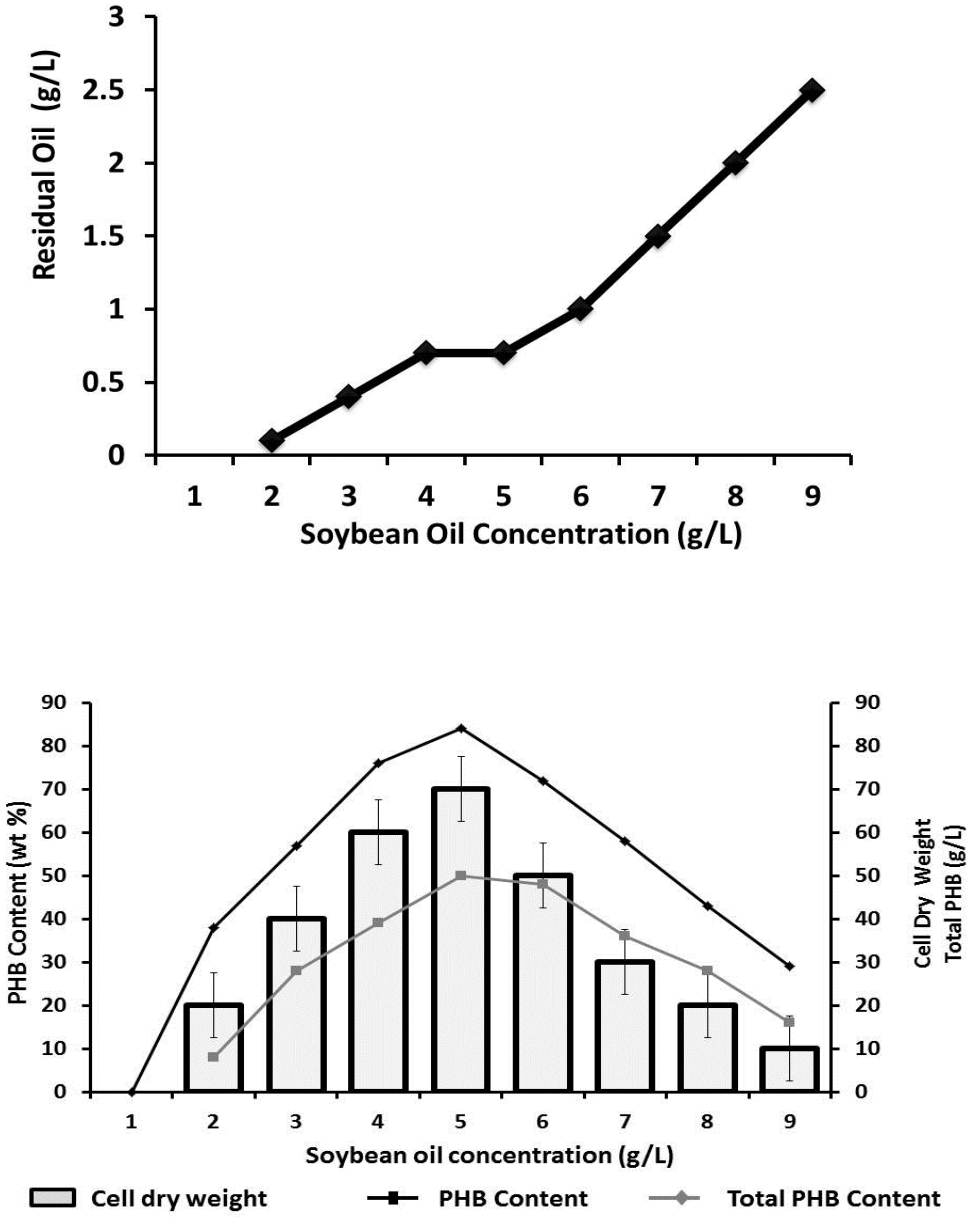

Figure 2 Effects of Soybean oil concentration on the biosynthesis of PHB by *Bacillus Subtillis*. The cultivation was conducted in 250 mL shake flasks, and incubated at 30°C at 200 rpm for 72 h. Data shown are the means of triplicate tests.

The processed soybean is the largest source of protein feed and second largest source of vegetable oil in the world. Soybean oil is widely used as edible oil whereas its meal is mainly used in animal feed industry. Direct consumption of soybeans is very limited. Around 35% of the beans production is traded in the world market [22].Research on Soybean has increased because this plant can thrive on most types of soil, but it thrives in warm, fertile, well-drained, sandy loam. However, the utility of soybean oil as the sole carbon feed for bacteria producing bioplastics has been proven by [23, 24].

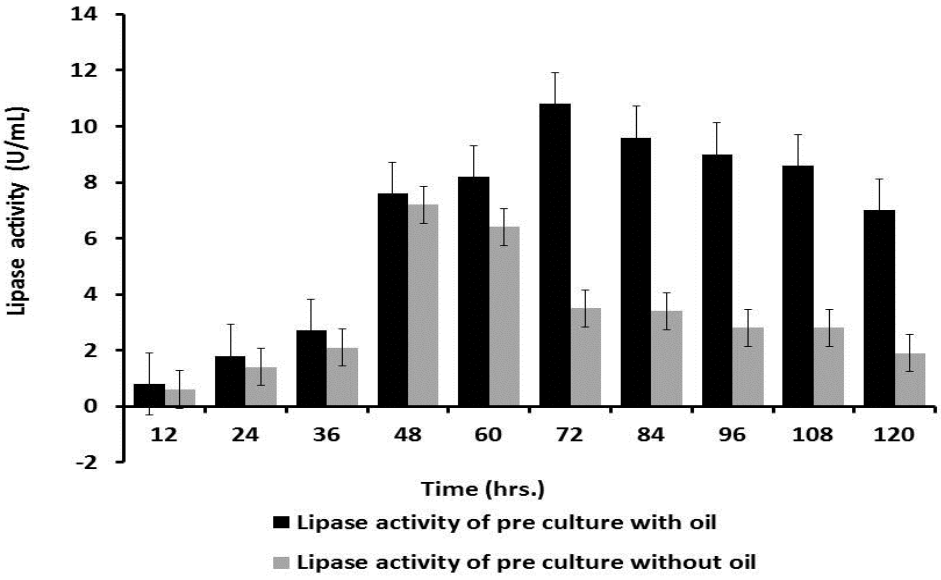

Fig.3. Effect of adding 1 g/L Soybean oil in preculture medium on lipase activity. The cultivation was conducted in 250 mL shake flasks, and incubated at 30 °C at 200 rpm for 72 hrs. Data shown are the means of triplicate tests. Mean data accompanied by different letters are significantly different [Tukey's HSD test, p <0.05].

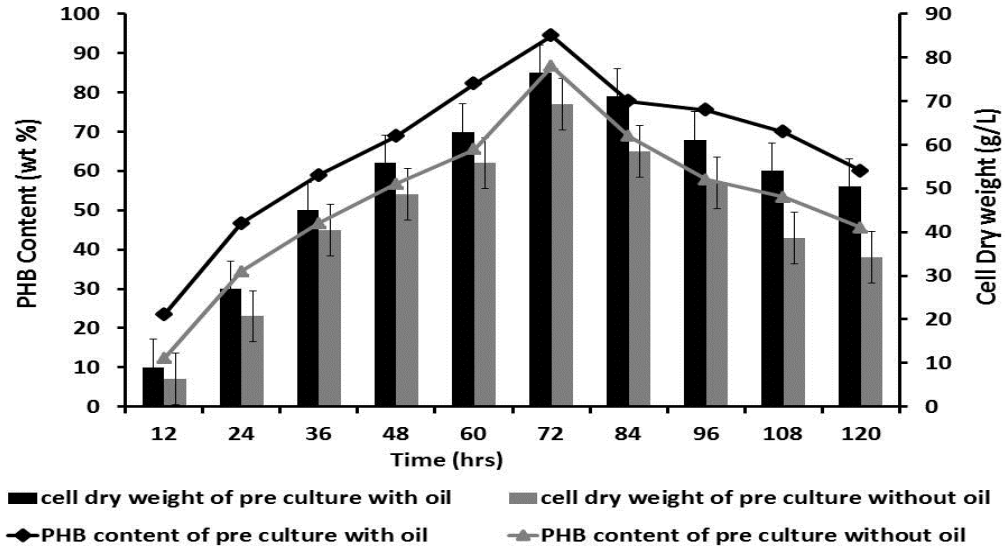

Figure 4 Effect of adding 1 g/L soybean oil in preculture medium on cell dry weight and PHA accumulation. The cultivation was conducted in 250 mL shake flasks, and incubated at 30 °C at200 rpm for 72 h. Data shown are the means of triplicate tests. Mean data accompanied by different letters are significantly different [Tukey's HSD test, p <0.05].

In current research, we investigated the potential of soybean oil in PHB production using *Bacilllussubtillis.* Apart from the advantages mentioned previously, we believe that Soybean oil has great potential as the sole carbon feed for bacteria producing PHA in India for several reasons. Firstly, India has potential for Soybean plantations because this plant grows more quickly and produces more seed in the tropics, especially on land with sufficient nitrogen and rainfall [25FAO, 2007]. Secondly, it was reported recently that soybean seed lipid content of 20 wt% of oil and Soybeans owe their dominance of the oil seed market to the value of their protein, which is much greater than that of the other oilseeds [26]. Soybean oil has 61% polyunsaturated fat and 24% mono unsaturated fat which is comparable

to the total unsaturated fat content of other vegetable oils [~ 85%] [8]. Here, we report for the first time the successful utilization of Soybean oil for producing PHB by *Bacillus subtillis.*

CONCLUSION

Soybean oil has been identified as a feasible and excellent carbon source for the production of PHB by *Bacillus subtillis*. This study also proved that the soybean oil do not affect cell growth and PHB production. Soybean oil is a potential carbon source to substitute for food-grade oils for large-scale production of PHA.

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