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Production of Biopolymers (Poly Beta Hydroxyl Butyric Acid) from *Bacillus cereus* SKC Strain

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Abstract: Our aim is to produce biopolymers by using *Bacillus cereus* isolates. And objectives are soil Samples were collected from industrial effluents of pydibhimavaram region, Srikakulam. Screening for PHB producing bacteria was done by serial dilution of various soil samples. PHB efficiencies of these isolates were analyzed by Law and Slepecky method. Efficient PHB bacilli were studied by standard morphological and biochemical methods for identification of the isolate as *Bacillus Cereus* SKC, which was found to be producing more PHB. Bacillus cereus isolated from industrial effluents of Pydibhimavaram region, Srikakulam was used in this study. Stock cultures were maintained at 4^0 C by periodic transfer on nutrient agar slants. Serial dilution then followed bymorphological tests like gram staining, Spore Staining Capsule Staining and various biochemical tests were performed for screening of isolates. The optimization of the physiological parameters i.e. the pH, Temperature, Carbon, Nitrogen, Phosphorous sources were performed for the enhanced production of Poly beta hydroxyl Butyric acid (PHB). The determination of the amount of PHB was performed chemically. Screening for PHB producing bacteria was done by serial dilution of various soil samples, collected from industrial effluents of pydibhimavaram region, Srikakulam, which resulted in isolation of four isolates. PHB efficiencies of these isolates were analyzed by Law and Slepecky method that resulted in selection of Gram positive endospore bearing bacillus. Efficient PHB bacilli was studied by standard morphological and biochemical methods that finally identified the isolate as *Bacillus Cereus* SKC , which was found to be producing more PHB. Culture conditions for better PHB yields by *Bacillus Cereus* SKC was studied by optimizing pH, temperature, nitrogen source, carbon source, phosphorous source. The results of this study concluded that the isolate is efficiently producing PHB at a pH 7.5 and temperature 40^0C. Among carbohydrate tested, glucose is found to be inducing better PHB production. Yeast Extract and Potassium dihydrogen phosphate was found to be best nitrogen and phosphorous sources for optimum PHB production. Finally, *Bacillus Cereus* SKC is suggested as another potential PHB producing microorganism.
Keywords: *Bacillus cereus*, pydibhimavaram, Srikakulam, biopolymers.

INTRODUCTION

The biodegradable polymer, PHB was first isolated and characterized in *Bacillus megaterium*, Later many other microorganisms were also reported to produce PHB as a carbon-energy storage material inside the cell under nutrient limited conditions, when a carbon source is readily available[1]. PHB exists as discrete granules in the cell making upto 70 – 80 % of dry cell weight and serves as reserves of carbon and nitrogen during stress conditions [2].

This biodegradable polymer has properties ranging from thermoplastics to elastomers and could potentially replace the harmful petroleum based plastics [3, 4]. Two major factors inhibiting wide spread use of PHB is the high investments in production and less rate of synthesis by microorganisms. The major limitation i.e lack of strength compare to chemical polymers was overcome by blending with other types of biopolymers [5]. For the economic production of PHB various Bacterial strains, either wild type or recombinant type and new fermentation strategies were developed for the production of PHB with high concentration and productivity [6]. Several works were reported for reducing the cost of PHB production using easily available and cheap raw materials [5]. Various research

groups have identified important proteins involved in biosysthesis and degradation of PHB. *Ralstonia eutropha,* the most extensivelystudied microorganism use 3-OH butyryl co-A to make PHB [7]. In during 1980 by means of Genetic engineering the 3 genes responsible for PHB production in *Alcaligenes Eutropha* were successfully transferred to the common bacterium *E.coli* making the way for recombinant bacterial systhesis.

Efficient and economic methods for recovery of PHB from recombinant *E. Coli* by simple digestion with chemicals was developed [8]. Over the past years PHB, co-polymers of PHB like PHB-HV, PHB-HP, and PHB-HH have been used to develop devices including sutures, repair devices, orthopaedic pins, stents, and tissue engineering devices bone marrow scafford agricultural and biomedical products and wound dressings [9]. In 1993 Mergaert team worked on biodegradation of PHB by microbial strain and found 295 microbial strains are capable of degrading PHB.

Within the last decade a team DOE plant research lab at Michigan state university, genetically modified plants to enable them to produce PHB. In 1992, they took the genes from PHB making Bacteria and inserted them directly into Cress plants and crossed them. Some of the off spring plants captured both the new genes and produced PHB in their leaves.

PHB is accumulated as energy reserve material in many prokaryotic organisms like *Alcaligenes, Azatobactor, Bacillus species, Pseudomonas species, Nocardia, Rhizobium et al.* [10]. A number of Bacillus species have been reported to accumulate PHB at 9 – 44.5 % dry cell weight [11].

The synthesis and break down of polyβ-hydroxy butyric acid in growing cultures of selected strains of *Rhizobium spirillum* and *Pseudomonas* have been studied [12]. They found 21.7 % of dry cell weight PHB in these organisms. *Spirillum* reported 34.2 % of PHB in Allison's medium containing Lactic acid as major carbon source.

By applying various mutagens like UV light, acriflavin and 5-bromo uracil to PHB producing B. sphaericusX3, B.subtilis K8, B.megateriumY6 and B. firmus G2, mutants with higher PHB production of about 70% has been reported. In this study a yield of 63.45 % was found in one mutant strain B.megaterium A13[13].

If a less expensive carbon source could be utilized, the overall cost of PHB production could be significantly reduced. In addition, much of the total cost of PHB production is attributable to purifying the PHB produced by the organism. Currently, PHB is purified by centrifugation, followed by mechanical lysis of the cells to release PHB, a high temperature procedure to agglomerate the PHB, and finally a spray-drying step to procure the purified granules.

In contrast to synthetic polymers, biopolymers tend to have a well-defined structure. Perhaps this is because evolution tends to select for chemical reactions and structures that are largely predictable. Biopolymers have an evenly distributed set of molecular weights and are built using a template-directed process.

MATERIALS AND METHODS

Our aim is to produce biopolymers by using Bacillus cereus isolates. And objectives are soil Samples were collected from industrial effluents of pydibhimavaram region, Srikakulam. Screening for PHB producing bacteria was done by serial dilution of various soil samples. PHB efficiencies of these isolates were analyzed by Law and Slepecky method. Efficient PHB bacilli were studied by standard morphological and biochemical methods for identification of the isolate as *Bacillus Cereus* SKC, which was found to be producing more PHB.

Bacillus cereus isolated from industrial effluents of Pydibhimavaram region, Srikakulam was used in this study. Stock cultures were maintained at 4^0 C by periodic transfer on nutrient agar slants. Serial dilution then followed bymorphological tests like gram staining, Spore Staining Capsule Staining and various biochemical tests were performed for screening of isolates. The optimization of the physiological parameters i.e. the pH, Temperature, Carbon, Nitrogen, Phosphorous sources were performed for the enhanced production of Poly beta hydroxyl Butyric acid (PHB). The determination of the amount of PHB was performed chemically

Microorganism

Bacillus cereus isolated from industrial effluents of Pydibhimavaram region, Srikakulam was used in this study. Stock cultures were maintained at 4^0 C by periodic transfer on nutrient agar slants.

Sterilization of Medium and glass ware

Sugars and mineral salts were autoclaved separately at 120^0 C for 10 lbs and15 lbs / $inch^2$ minutes respectively and mixed together after attaining the room temperature. The glass ware like conical flasks, pipettes, test tubes and measuring cylinders were autoclaved at 15 lbs / $inch^2$ pressure for 15 minutes.

Inoculum preparation

Inoculum was prepared in 250 ml conical flasks containing 50 ml sterile medium consisting nutrient broth with 10g / lit of sucrose and the pH was adjusted to 7.0. Flasks were incubated at 35 ± 0.5^0 C for overnight on a rotary shaker at 250 rpm / min.

Isolation of bacteria from soil polluted with industrial effluent

Serial dilution

Collected soil samples are taken and test tubes containing 9ml of sterile saline water and sterile Petri dishes were taken and labelled accordingly ($10^{-2,}$ 10^{-3}…10^{-9}) with a marking pencil. Then 1 gm of sample of fine pulverized, air dried soil was added into 9ml sterile saline water to make 1:10(10^{-1})**.** The dilution was vigorously shaken to obtain uniform suspension of microorganisms. 1ml of suspension was transferred from the 1st test tube into 2nd test tube with a sterile pipette under aseptic conditions to make 1:100(10^{-2}) dilution and shake it well. Another dilution 1:1000 was prepared by pipetting 1ml suspension from 1:100 dilution tubes into 3rd test tube. Further dilutions 10^{-4} to 10^{-9} were made, by pipetting 1ml suspension into remaining test tubes. Nutrient media for the organisms was prepared as per composition and sterilized in autoclave at 121°c, 15 lbs pressure for 15 mins. The sterilized media was transferred into Petri dishes and was allowed to solidify. 0.1ml of aliquots from 10^{-6}, 10^{-7}, and 10^{-8} was transferred to different Petri dishes and three control Petri dishes were also maintained. The plates were incubated at 30°c for 72 hrs and plates were observed after 24 hours of incubation for growth. The pure cultures obtained (of the bacterial species) were cultured in LB medium by incubating at 37°cfor 24 to 48 hrs in the form of agar plates, slants, and broth.

Streak Plate Technique

Petri dishes containing nutrient agar growth medium was prepared and solidified. Flames sterilize a wire loop. Using aseptic technique, the sterile loop was used to make parallel streaks of the suspension on the agar. (Note: there should be 16 streaks, 4 sets of 4, and whole surface of plate should be used). The plates were covered and. inverted for incubating it under low light at constant temperature. The colonies were selected. For further isolation, the sample was taken using a sterilized bacteriological loop and was placed in a drop of sterile culture medium on a glass slide. The morphology of the desired species was checked microscopically. Selection of PHB producing isolates was done by staining the colonies with Nile blue. The PHB producing isolates were picked and were cultured in the plates containing NAM media separately and incubated at 37^0 C for 24 h. All the above cultured plates were subjected to PHB estimation by Crotonic acid method.

S .No	ISOLATES	PHB PRODUCED
01	Isolate no 1	-
02	Isolate no 2	++
03	Isolate no 3	+++
04	Isolate no 4	+

NOTE: -

1.- → NO PRODUCTION OF PHB

2.+ → LESS PRODUCTION OF PHB

3.++ →SATISFIABLE PRODUCTION OF PHB

4.+++→GOOD PRODUCTION OF PHB

The isolate no 3 gave good results and hence was selected for the further studies.

MORPHOLOGICAL TEST

Gram Staining

Thin smear of culture was prepared on a clean glass slide and the smear was air-dried. The smear was heat fixed. The smear was flooded with crystal violet for 30 seconds. The slide was washed with the distilled water for a few seconds, using wash bottle. The smear was covered with Gram's iodine solution for 60 seconds. The iodine solution was washed off with 95% ethyl alcohol and ethyl alcohol was added drop by drop, until no more color flows from the smear. The slide was washed with distilled water and drained. Safranin was added to the smear for 30 seconds (counter staining). The slide was washed with distilled water and dried completely using a blotting paper. The slide was observed microscopically using oil immersion.

Spore staining

A smear was prepared and fixed with 20 passes through a flame. The smear was placed on a screen well above a moderate flame from a Bunsen burner. A generous amount of saturated aqueous malachite green was applied to the slide and was allowed to steam off for 10 to 20 minutes. Ideally, the heat was maintained so that the dye barely steams, that is, whitish vapors are barely visible. After cooling the slide was rinsed with tap water to remove excess stain. The slide was then counterstained for a minute in 0.25% aqueous safranin. The slide was then rinsed, blotted, and dried.

Capsule staining

A drop of broth culture was placed on one end of a microscope slide. One drop of Indian ink was added and left for one minute. A second slide was taken and and it was held at 450 to make a thin layer by a continuous forward movement. The film was air dried and was observed under microscope.

BIOCHEMICAL TESTS

Catalase Activity

The contents were mixed thoroughly and the medium was sterilized by autoclaving at 15lbs pressure for15 minutes. After autoclaving the medium was cooled and then poured into two sterile test tubes, and was allowed to solidify in a slanting position. One of the tryptone soya agar slants were inoculated with culture. And another uninoculated one was kept as a control. The cultures were incubated at 37^{o}C for 48 hours. About few drops of Hydrogen peroxide was added.

Hydrogen Sulphide Production Test

6.5g of TSI media was weighed (make HI-MEDIA) and dissolved in 100 ml of Distilled water; and sterilized at 121^{o}Cfor 15 min. After autoclaving the medium was allowed to solidify. One of the tubes was inoculated with culture, and the other one was kept as a control. The tubes were incubated at 35-36^{o}C for 48 hours.

Indole Production Test

Preparation of Tryptone and then broth was cooled and poured in the sterile test tubes. One of the tubes was inoculated with culture, and the other one was kept as a control. The tubes were incubated at 35-36^{o}C for 48 hours. About 1 ml of the Kovacs reagent was added.

Methyl-Red and Voges- Proskauer Tests

Preparation of MRVP broth (pH 6.9) and then sterilize by autoclaving at 15lb pressure for 15 minutes. After autoclaving cool to 50^{o}C and then pour into four sterile test tubes. Inoculate three test tubes with culture and keep one tube as inoculated comparative control. Incubate all four tubes at 35^{o}C for 48 hours. Add 5 drops of methyl red indicator to two tubes.

Citrate Utilization Test

3.4g of Simmon's citrate agar was weighed (make HI-MEDIA) and dissolved in 100 ml of Distilled water; and sterilized at 121^{0}Cfor 15 min. After autoclaving the medium was allowed to solidify. One of the tubes was inoculated with culture, and the other one was kept as a control. The tubes were incubated at 35-36^{0}C for 48 hours.

Urease Test

2.8 g of Urea was weighed (make HI-MEDIA) and dissolved in 100 ml of distilled water; and sterilized at 121^{o}Cfor 15 min. After autoclaving the medium was allowed to solidify. One of the tubes was inoculated with culture, and the other one was kept as a control. The tubes were incubated at 35-36^{o}C for 48 hours. About 3 ml of Phenol red was added to the culture.

Starch Hydrolysis

The NAM media was prepared for about 50 ml. The medium was cooled and poured into two sterile test tubes, and was allowed to solidify. One of the agar slants were inoculated with culture by means of a stab-and-streak inoculation and another tube is kept as an inoculated comparative control. All the tubes were incubated at 37^{0}C for 48 hours and few drops of Iodine solution was added after the incubation period.

Carbohydrate Fermentation

The Glucose broth was prepared for about 50 ml. The medium was cooled and poured into two sterile test tubes, and was allowed to solidify. One of the agar slants were inoculated with culture by means of a stab-and-streak inoculation and another tube is kept as an inoculated comparative control. The Durham's tubes were inserted without the formation of bubbles. All the tubes were incubated at 37^{0}C for 48 hours and few drops of Methyl red was added after the incubation period.

MOLECULAR TESTS

Curing of Plasmid DNA from the Bacteria

To detect whether PHB producing genes are Plasmid borne or main chromosomal borne. Curing of the plasmid DNA was done to remove the plasmid from the cells. To remove the plasmid, curing agents like Sodium lauryl sulphate, Sodium dodecyl sulphate, Acridine orange, Mitomycin-C, Ethidium bromide can be used at various concentrations. For this study Sodium lauryl sulphate, ethidium bromide was used for curing plasmid from the bacterium. Fresh cultures of overnight incubated bacterium was taken and the organism was inoculated in the LB-Broth along with the Sodium lauryl sulphate at increasing concentrations of 100 µg/ml. 125 µg/ml., 500µg/ml., 750 µg/ml and in the ethidium bromide at 10 µg/ml and 25 µg/ml All the flasks were incubated in the orbital shaker at 200 rpm at 30 C for 48 hrs. A concentration that is below MIC (Minimum Inhibitory Concentration) was selected as curing concentration.

Optimization of physical and nutrient parameters for enhanced PHB production

The various parameters selected for this study are temperature, pH, carbon, nitrogen, phosphorus. All experiments were conducted in duplicates and repeated twice. The results presented in the results section are mean of the obtained values.

Effect of pH

Effect of various pH levels on PHB production was observed by incubating the culture broth at pH levels ranging from 6 to 8 with a difference of 0.5. The different pH levels were adjusted using 2N NaOH (Sodium Hydroxide) to the 50 ml production medium taken in 250 ml conical flask, inoculated with active culture of *Bacillus Cereus* SKC and incubated at 37^{0} C for 48 hours. The PHB production was estimated at an interval of 24th and 48th hour using the procedure mentioned earlier.

Effect of temperature

Effect of various temperatures on PHB production was observed by incubating the active culture broth at various temperatures 25^0 C, 30^0 C, 35^0 C, 40^0 C, 45^0 C and 50^0 C for 48 hours. The cell suspension was collected at an interval of 24^{th} and 48^{th} hour and the PHB production was estimated with the procedure mentioned earlier.

Effect of various carbon sources

The effect of various carbon sources on PHB production was observed by incubating the culture in 50 ml production medium-1 containing various carbon sources like Sucrose, Glucose, Galactose, Maltose, Lactose, Xylose etc. in 250 ml conical flasks, incubated with active culture of *Bacillus cereus* SKC strain. The incubation was carried out for 48 hour and the PHB production was estimated at an interval of 24^{th} and 48^{th} hour using the procedure mentioned earlier.

Effect of Nitrogen sources

The influence of various nitrogen sources on PHB production was investigated by using different nitrogen sources at a concentration of 2gm /l in the medium. The various nitrogen sources tested are Casein, Peptone, Yeast extract, Potassium nitrite, Malt extract and Urea. The production medium with these nitrogen sources was inoculated with active culture of *Bacillus cereus* SKC and incubated at 37^0C for 24 hrs on shaker at 250 rpm / min. The PHB production was estimated at an interval of 24^{th} and 48^{th} hour by using the procedure mentioned earlier.

Effect of various Phosphorous sources

The Influence of various phosphorous sources on PHB production was investigated by using various phosphorous sources at a concentration of 1gm/l. The various phosphorous sources used are Na_2HPO_4, K_2HPO_4, NaH_2PO_4 and KH_2PO_4. The production medium with these phosphorous sources was inoculated with active culture of *Bacillus cereus* SKC incubated at 37^0C for 48 hrs. And the PHB production was estimated at 24^{th} and 48th hour using the procedure mentioned earlier.

Estimation of PHB

The optimization of the physiological parameters i.e. the pH, Temperature, Carbon, Nitrogen, Phosphorous sources were performed for the enhanced production of PHB. The determination of the amount of PHB was performed chemically. The microorganism Bacillus Cereus SKC was grown on the Nutrient Broth medium at 400 C, pH 7.5 for 48 h. Suspensions of cultures were centrifuged at 3000 rpm for 15 min. The Wet weight and the Dry weights were recorded simultaneously. Then the pellets were suspended in 3ml of sodium hypochlorite solution and kept for 12 h incubation. After the incubation the contents were centrifuged again at 3000 rpm for 15 min. Next the granules were washed thoroughly with acetone: alcohol (1: 1). The residue was dried and the polymer was extracted into boiling chloroform. After evaporation of the chloroform five milliliters of conc. Sulphuric acid was added. The tubes were heated at 1000 C in a water bath for 10 min. After cooling to 250 C, the amount of PHB was determined on a U.V. Spectrophotometer, wavelength 235 nm [15, 14]. Some of the A. eutrophus strains used for commercial PHB production has a PHB concentration which is approx 80 % (w/w) of the dry cell weight [4].

Estimation of Proteins by Lowry Method

5ml of the alk. Solution was added to 1ml of the test solution. The Solution was mixed thoroughly and was allowed to stand for 10 min. 0.5 ml of diluted Folin-Ciocalteau reagent was added rapidly with immediate mixing. After 30 min the absorbance was read against the appropriate blank at 750 nm.

RESULTS & CONCLUSION

Screening for PHB producing bacteria was done by serial dilution of various soil samples, collected from industrial effluents of pydibhimavaram region, Srikakulam, which resulted in isolation of four isolates. PHB efficiencies of these isolates were analyzed by Law and Slepecky method that resulted in selection of Gram positive endospore bearing bacillus. Efficient PHB bacilli was studied by standard morphological and biochemical methods that finally identified the isolate as *Bacillus Cereus* SKC, which was found to be producing more PHB. Culture conditions for better PHB yields by *Bacillus Cereus* SKC was studied by optimizing pH, temperature, nitrogen source, carbon source, phosphorous source,. The results of this study concluded that the isolate is efficiently producing PHB at a pH 7.5 and temperature 40^0C. Among carbohydrate tested, glucose is found to be inducing better PHB production. Yeast Extract and Potassium dihydrogen phosphate was found to be best nitrogen and phosphorous sources for optimum PHB production. Finally, *Bacillus Cereus* SKC is suggested as another potential PHB producing microorganism.

REFERENCES

1. Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological reviews. 1990 Dec 1;54(4):450-72.
2. Merrick, J. M., Delafield, F. P., & Doudoroff, M. (1962, January). Hydrolysis of poly-beta-hydroxybutyrate by intracellular and extracellular enzymes. In *federation proceedings* (vol. 21, no. 2, p. 228). 9650 rockville pike, bethesda, md 20814-3998: federation amer soc exp biol.
3. Bichler C, Drittler R, Langowski HC, Starnecker A. Biologisch abbaubare Verpackungsmaterialien-die Loesung des Muellproblems?. Bioengineering-grafelfing-. 1993;9:12.

4. Lee SY. Bacterial polyhydroxyalkanoates. Biotechnology and bioengineering. 1996 Jan 5;49(1):1-4.
5. Baltieri RC, Mei I, Lucia H, Bartoli J. Study of the influence of plasticizers on the thermal and mechanical properties of poly (3-hydroxybutyrate) compounds. InMacromolecular Symposia 2003 Jul 1 (Vol. 197, No. 1, pp. 33-44). WILEY-VCH Verlag.
6. Lee PC, Lee WG, Lee SY, Chang HN. Succinic acid production with reduced by-product formation in the fermentation of Anaerobiospirillum succiniciproducens using glycerol as a carbon source. Biotechnology and Bioengineering. 2001 Jan 5;72(1):41-8.
7. Pötter M, Madkour MH, Mayer F, Steinbü CHEL A. Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in Ralstonia eutropha H16. Microbiology. 2002 Aug 1;148(8):2413-26.
8. Choi J, Lee SY. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Applied Microbiology and Biotechnology. 1999 Jan 1;51(1):13-21.
9. Chen GQ, Wu Q. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials. 2005; 26(33):6565-78.
10. Braunegg G, Lefebvre G, Genser KF. Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. Journal of Biotechnology. 1998 Oct 27;65(2-3):127-61.
11. Lee YW, Yoo YJ. Kinetics for the Growth of Alcaligenes eutrophus and the Biosynthesis of Poly-${\beta} $-hydroxybutyrate. Microbiology and Biotechnology Letters. 1991;19(2):186-92.
12. Hayward AC, Forsyth WG, Roberts JB. Synthesis and breakdown of poly-β-hydroxybutyric acid by bacteria. Microbiology. 1959 Jun 1;20(3):510-8.
13. Hikmet Katyrcyoolu, Belma Aslym, Zehra Nur Yuksekdao, Nazime Mercan, Yavuz Beyatly. Production of Poly_hydroxybutyrate 9PHB) and differentiation of putative Bacillus mutant strains by SDS-PAGE of total cell protein. 2003;149.
14. Aslım B, Çalışkan F, Beyatlı Y, Gündüz U. Poly-β-hydroxybutyrate production by lactic acid bacteria. FEMS microbiology letters. 1998 Feb 1;159(2):293-7.
15. Katırcıoğlu H, Aslım B, Yüksekdað ZN, Mercan N, Beyatlı Y. Production of poly-β-hydroxybutyrate (PHB) and differentiation of putative Bacillus mutant strains by SDS-PAGE of total cell protein. African Journal of Biotechnology. 2003;2(6):147-9.