



Production of Polyhydroxybutyrate (PHB) from *Streptomyces incanus* and the Effect of Gamma Irradiation

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Polyhydroxybutyrate (PHB) is a biosynthetic and biodegradable plastic which attracts attention due to its properties. Out of nine Actinomycetes isolates obtained from different soil samples, the PHB accumulating isolate was identified by molecular studies and was identified as *Streptomyces incanus* BK128. Different agricultural waste (wheat bran, rice bran, rice straw and molasses) were employed for polyhydroxybutyrate (PHB) production as the main carbon source. Some supplements such as (0.4 g) ammonium sulphate were used to optimize the PHB production medium. The maximum PHB production was achieved with wheat bran (1.9g/l). Different parameters including; the initial pH of the medium, ammonium chloride concentration and inoculum size; were carried out under shaking flask conditions (150 rpm) at 30°C for 5 days of incubation to enhance the PHB production. The maximum PHB production (2.82g l⁻¹) was achieved at pH 6.5, (2%) NH₄CL and 5 ml inoculum size, respectively. The effect of gamma irradiation on the activity of *Streptomyces incanus* BK 128 towards PHB production showed that, at dose level 1.5 kGy, the maximum PHB production obtained was 3.39 g l⁻¹.

Keywords: Biopolymer /PHB/ *Streptomyces incanus* / Wheat bran/ rice bran

Introduction

Amongst the various existing biodegradable plastic materials, PHB (polyhydroxybutyrate) has achieved foremost consideration due to their structural variety and close correlation to plastics. The fact that PHB can be produced from renewable resources, and their absolute biodegradability quality, makes them the most hopeful product in the near future. PHB has been produced in different scales via fermentation processes as eco- friendly and sustainable plastics [1].

Environment contains abundance of lignocellulosic waste materials and agricultural residues like rice straw, wheat straw, rice bran etc., which could be used for economic PHB production. The major obstruction for the exploitation of lignocellulose is the high stability of this material. Large amounts of lignocellulosic biomass (LB) generated from several agricultural and forest activities may provide inexpensive renewable resources [2].

Molasses is a carbon source for PHB production, and the presence of growth factors especially organic acids, vitamins and minerals in molasses may enhance PHB yield [3].

The importance of strain improvement could be achieved either through hybridization and selection within natural population or through population of induced mutant. However, induction of mutation followed by selection has proved to be the most efficient programs for the isolation of highly productive cultures [4]. Bibers and Kalnis, [5] proved that gamma irradiation of mutant of *Streptomyces* sp. at a dose level of (0.25 – 1.5) KGy significantly enhanced PHB production.

Therefore, the present study was carried out to produce PHB from a cheap carbon source (waste product agriculture) such as wheat bran, rice straw, molasses and rice bran, and to determine the influence of nutritional conditions as well as the effect of gamma irradiation on the PHB accumulation by the most active organism.

Materials and Methods

Isolation and selection of Actinomycetes:

Seven Rhizosphere soil samples (lettuce, garlic, onion, zucchini, Eggplant, wheat and alfalfa) were used to isolate Actinomycetes which have the ability to produce PHB using starch nitrate agar medium. For maintenance, the isolates were inoculated into nutrient agar slants and incubated at 30°C for 5 days before storage in a refrigerator at 4°C for further examination.

Estimation of PHB Production by Staining:

The estimation of PHB was carried out by staining the whole bacterial colonies grown on petri-dishes using Sudan Black B and its modification [7]. Then, the most producer isolate was genetically identified.

Identification of the most active organism

To characterize the genus of *Actinomycete* selected as PHB producer, gene-based 16SrRNA identification was used. Molecular identification of the *Streptomyces* isolate with the highest production of PHB was made. Pure mature culture of the organism for further molecular taxonomic identification using 16S rRNA and primers of the selected isolate according to the National Center for Biotechnology Information (NCBI). This test was conducted in Sigma Laboratory, Cairo, Egypt according to method described by Kaiser et al.[6]. DNA extraction was made according to the Protocol of Gene Jet genomic DNA purification Kit (Thermo K0721). The 16S rRNA gene sequence analysis was carried out using NCBI-

BLAST (the National Center for Biotechnology Information) program.

Cultural Conditions:

For the fermentation in a 250 ml flask culture technique, the standard inoculum of seed culture was added into 100 ml starch nitrate broth medium and incubated at an agitation speed of 150 rpm at 30°C for 48 h in a rotary incubator shaker to initiate the growth [8].

Determination of PHB production:

PHB polymer was extracted following the technique previously described by Law et al. [12] and the amount produced was calculated from the standard curve prepared by using commercial poly-β-hydroxybutyrate (Sigma-Aldrich) [13]. The PHB content was defined as the ratio of PHB concentration to cell concentration given in as a percentage.

PHB Production Parameters:

Batch fermentations were carried out in 250 ml Erlenmeyer flasks containing 100 ml of culture medium. The flasks were inoculated by the tested organism and maintained at 30°C and 150 rpm for 5 days. A set of experiments were performed at different pH values (5.5-7.5), inoculum size (2-7 ml) and NH₄CL concentration (1-4%) to investigate the effect of these parameters on the PHB production and growth.

Effect of different substrates on PHB production:

A concentrated rice straw solution was used. The solution was prepared according to the technique reported by Sindhu et al. [9]. For pretreatment, the distracted wheat bran (WB) and rice bran were mixed with 1% NaOH as reported in a previous study [10]. Molasses were used at 20 g/l as a carbon source with or without the addition of 0.8 g/l ammonium sulphate as nitrogen sources [11], and the yield of PHB as well as the growth of *Streptomyces incanus* BK128 were estimated after 5 days of incubation.

Effect of Gamma Irradiation on production of PHB:

The effect of different doses of gamma irradiation (0.25-3.5 kGy) on the growth and PHB production were investigated. Irradiation was carried out at the National Center for Radiation Research and

Technology (NCRRT), using ^{60}Co gamma irradiation source of Russian facility with a dose rate 1.812 kGy/ min at the time of the experiment.

Results and Discussion

From the obtained 48 isolates which were tested for their ability to produce PHB, nine isolates show the ability to produce PHB. The most active one was genetically identified based on 16SrRNA and showed 100% of similarity to the genus *Streptomyces incanus* BK128.

Effect of pH on growth and PHB produced by Streptomyces incanus BK128.

Optimization of batch culture conditions in order to enhance PHB accumulation by *Streptomyces incanus* BK128 has been studied. The data in Fig.1 revealed that *Streptomyces incanus* BK128 was able to grow to different degrees in all pH values from acidic to alkaline starch nitrate medium. The maximum growth and PHB accumulation was achieved at initial pH 6.5 which recorded 4.47 and 2.23 g l^{-1} respectively. PHB yield at extreme acidic pH and extreme alkaline pH was dramatically decreased. Change in medium pH may influence the growth of the bacterium, and would influence the PHB production. Typically, a metabolic process may be extremely prone to even minor changes in pH and therefore, proper control of pH is critical for PHB production. Hence, PHB production is sensitive to the pH of cultivation. The most favorable pH for bacterial growth is neutral pH (6.5-7.0), and hence contributes to higher PHB production [14].

Effect of inoculum size on growth and PHB produced by Streptomyces incanus BK128.

The effect of the inoculum size on the growth and PHB production was studied. The starch nitrate media were inoculated with different inoculum size (2-7 ml), incubated at 150 rpm and 30°C for 5 days. The data represented in Fig.2 illustrated that the cell growth increased from 2.57 to 4.25 g/l by increasing the inoculum size from 1 to 5 ml. Further increase in inoculum size lead to a slight decrease in cell concentration. On the other hand, the PHB production increased gradually from (0.98 to 2.43 g/l) by increasing inoculum size from 1 to 5 ml, respectively. Then PHB concentration began to decrease slightly by further increase of

inoculum size reaching to 1.85 g/l by adding 7ml inoculum.

Effect of NH_4CL concentrations on growth and PHB produced by Streptomyces incanus BK128.

The effect of different concentrations of NH_4CL (1-4%) on both growth and PHB accumulation was also studied. The data in Fig. 3 revealed that the growth decreased with increasing NH_4CL concentration from (3-4%). The maximum growth and PHB production (4.84 g l^{-1} , 2.82), respectively was obtained by adding 1.25 % NH_4CL to the tested medium, and then decreased with further increase in NH_4CL concentration.

Low nitrogen concentration is good for PHB production. Under normal conditions, bacteria synthesize their cell materials like proteins and can grow well. But in nutrient limiting conditions bacteria may shift their protein synthesis to PHB synthesis for survival. Therefore, nutrient control and environmental stress are required to prompt PHB production. PHB production needs a high carbon source in addition to a nitrogen limitation [15]. Similarly, Thakur et al.[16] worked on the production of PHB and obtained the highest PHB yield in ammonium chloride followed by ammonium sulphate.

Effect of different substrates on growth and PHB produced by Streptomyces incanus BK128.

Waste materials are frequently important for economic and environmental reasons. The biotechnological utilization of these wastes will eliminate a source of pollution and the products will be obtained cheaply at the same time. For this reason molasses, wheat bran and rice bran were used in the present study as the main carbon source in the production medium. The data given in Fig.(4) demonstrated that the maximum growth was obtained by wheat bran medium (3.9 g/l) followed by rice bran (2.22 g/l). While, the maximum PHB production was obtained by wheat bran medium (1.9 g/l) followed by rice bran medium with PHB production (0.95g/l). On the other hand, molasses medium recorded unexpectedly the lowest growth and PHB synthesis (0.15 and 0.03g/l respectively).

The obtained results indicated that, the maximum PHB production was obtained by wheat bran medium with high PHB content. PHB production had been reported from several bacteria by using waste materials or agricultural residues as

inexpensive carbon sources [17]. Furthermore, readily available low-price carbon sources may have high amounts of nutrients, such as amino acids and peptides, which contributes to improved cell growth and metabolite biosynthesis and pave the way for resourceful and cost-effective production of PHB. PHB being the reserved food polymer produced during time of starvation, is degraded to provide carbon and energy when external carbon source is exhausted [18].

Various waste materials have also been considered as potential carbon sources for PHB production. Low cost agro-industrial residues like, wheat bran, rice bran have been investigated for PHB production by various researchers [3-10]. Molasses, which is a common industrial by-product from sugar industries, is rich in nutrients, minerals and at the same time it is cheap, available in plentiful amounts and could be exploited for production of variety of industrial products [19].

Effect of gamma irradiation on the activity of Streptomyces incanus BK128 towards PHB production.

The effect of gamma irradiation on the activity of *Streptomyces incanus* BK128 towards PHB production was carried out. The strain was exposed

to increasing doses of gamma radiation (0.25 to 3.5 kGy). The cell concentration, PHB concentration and PHB content were investigated. The data in Fig. (5) shows that low doses of gamma radiation from 0.25 to 2 kGy may stimulate both growth and PHB production. The highest cell concentration and PHB concentration 5.54 and 3.39 g l⁻¹ were achieved at the dose 1.5 kGy, respectively with PHB content of 61.2%. However, a decrease in the cell concentration accompanied by low PHB production was achieved by the increasing doses of gamma radiation (>2 kGy). Several studies recorded that low doses of gamma radiation may stimulate the microbial growth and metabolic activities [20-21]. Meanwhile, high doses of gamma radiation were proved to be inhibitory for both growth and enzymatic activities of microorganisms. The exposure of cells to ionizing radiation sets off a chain of reactions giving rise to chemical changes followed by metabolic or physiological changes. The irradiation presents an additional stress to the cells which tends to disturb their organization [22]. Irradiation effects have been shown to occur with proteins, enzymes, nucleic acid, lipids and carbohydrates, all which may have marked effects on the cell.

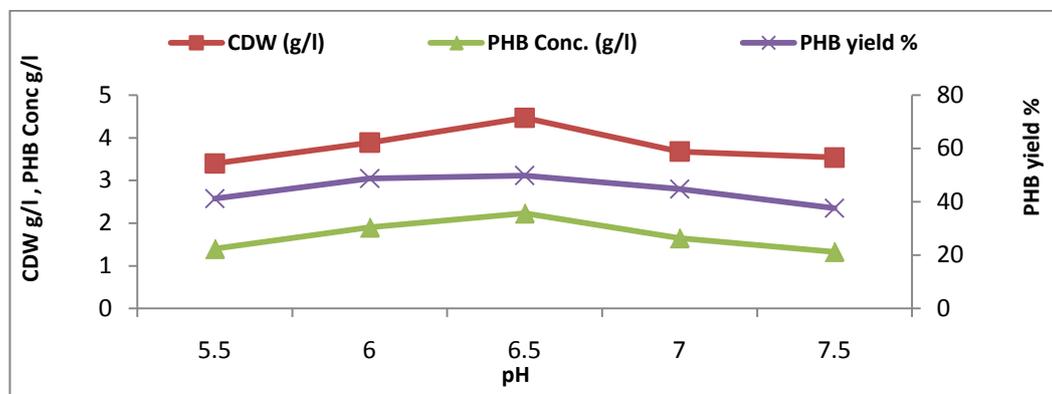


Fig. (1): Effect of pH on growth and PHB production from *Streptomyces incanus* BK128.

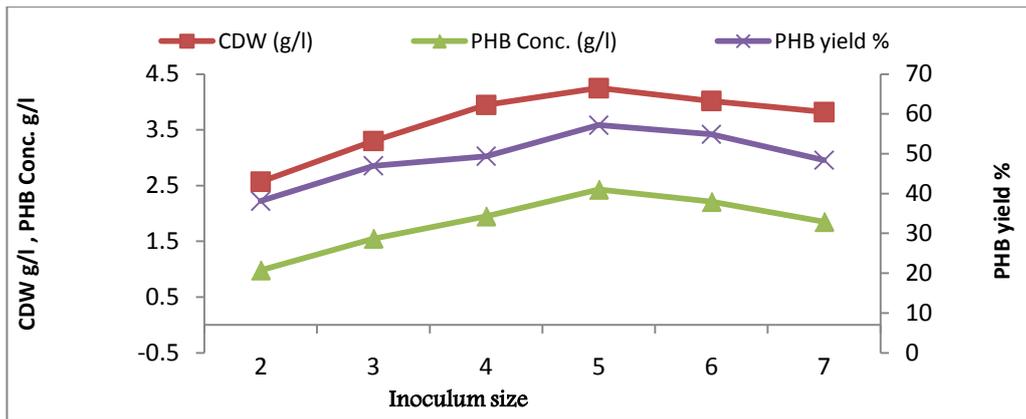


Fig. (2): Effect of inoculum size on growth and PHB production from *Streptomyces incanus* BK128.

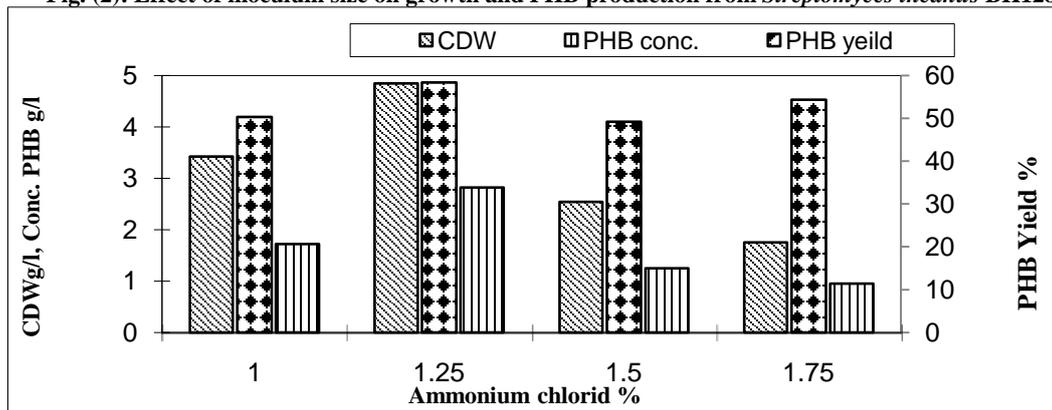


Fig. (3): Effect of NH₄CL concentrations on growth and PHB production from *Streptomyces incanus* BK128.

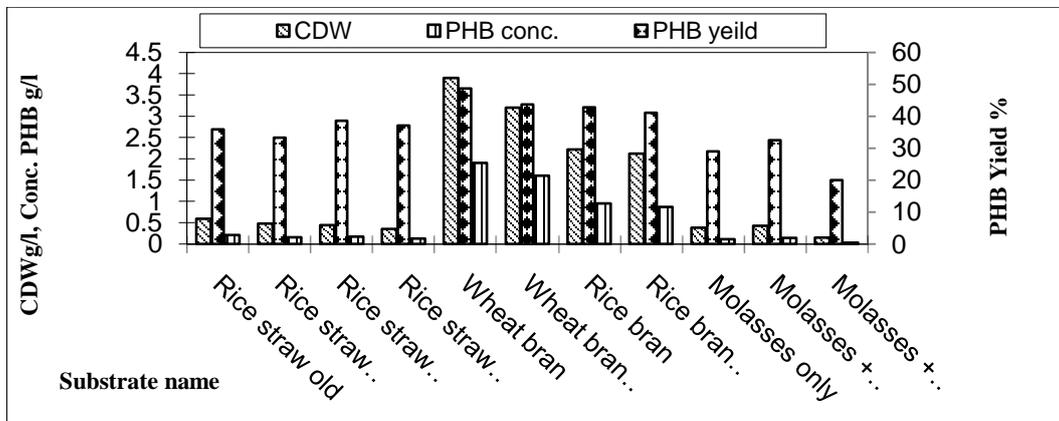


Fig. (4): Effect of different substrates on growth and PHB production from *Streptomyces incanus* BK128

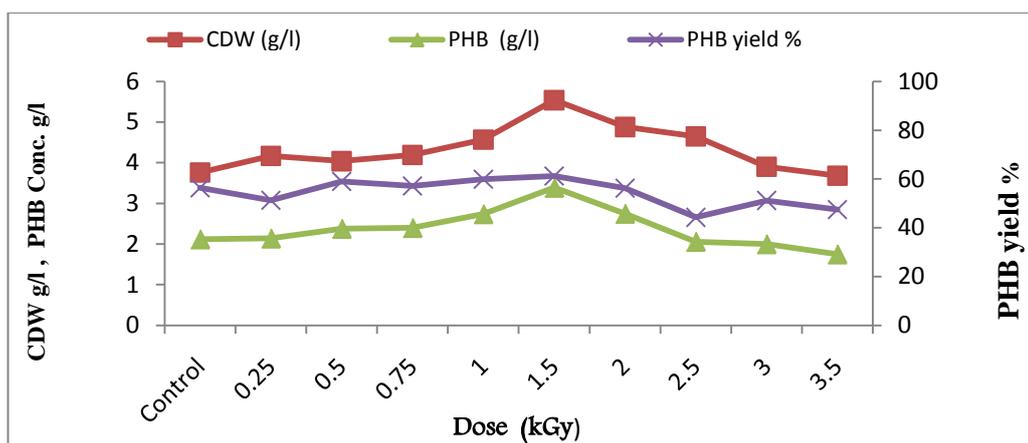


Fig. (5): Effect of gamma irradiation on the activity of *Streptomyces incanus* BK128 towards PHB production

Conclusion

This study proved that wheat bran can be used as an alternative to starch nitrate medium for PHB production. Optimization of flask culture conditions indicated that the maximum PHB production by *Streptomyces incanus* BK128 could be achieved on 100 ml of starch nitrate medium with initial pH 6.5 at 30 °C for 5 days, 150 rpm rotary shaker, and 5ml inoculum's size. These conditions resulted in 4.84 and 2.82 gl^{-1} of cell dry weight and PHB dry weight, respectively with 56.9% of PHB content. However, both growth and PHB production were found to be stimulated by the exposure of *Streptomyces incanus* BK128 to low doses of gamma radiation (0.25–2 kGy). The highest cell concentration (5.54 gl^{-1}) and PHB accumulation (3.39 gl^{-1}) was achieved at the dose 1.5 kGy with PHB content 61.2%.

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