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Microbial Production of Poly-3-Hydroxyl Butyrate

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ABSTRACT

Economics of production of bioplastics mainly affects on feasibility for its adequate use to fulfill the actual needs in day today life. Cost of media used in the production play a critical role in overall economics. Whey waste of dairy industry provide ample amount of carbohydrates and proteins as well as lipids and fats. Production of PHB observed on whey waste sample was approximately 0.12 gm per gm of biomass. Acrylic acid blocks the beta oxidation of fatty acid degradation. Accumulation of fatty acid in larger amounts inside the cell results in to the production of polyhydroxy butyrate in superior amounts due to the diversion of intermediates towards the polyhydroxy butyrate production. As the isolate were obtained from nutrient deficient and stress condition they are having better ability to accumulate the nutrients during its stationary phase or starvation conditions for its survival. When these isolates are grown in an optimum condition with suitable nutrients gives good polyhydroxy butyrate production. Acrylic acid concentration above the optimum level is inhibitory for its growth so it is optimized on a range of 10µl/lit to 250µl/lit. Its optimum concentration gives sufficient growth and maximum polyhydroxy butyrate production. Physiological conditions such as pH, temperature also affect the fermentation processes that are optimized for maximum production of polyhydroxy butyrate. It should be noted that organisms which are isolated from stress condition and capable of tolerating the acid concentration gives superior production of polyhydroxy butyrate by mentioned strategy.

Key words: - Polyhydroxy butyrate, bioplastics, acrylic acid, whey waste.

1. INTRODUCTION

Polyhyhydroxy butyrate (PHB) is a Poly hydroxyalkanoate (PHA), a polymer belonging to the Polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non biodegradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental waste management problem emanating from plastic materials. Bioplastics are having properties like biodegradability and renewable so environmentally safe. A fully biodegradable polymer is defined as a polymer that is completely converted by living organisms, usually microorganisms, to carbon dioxide, water and humic material. Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends[1].Biopol [poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)], a copolymer of monomers of 3-

hydroxybutyrate (HB) and 3-hydroxyvalerate (HV), is a biodegradable PHA .Thermoplastic that was produced by Imperial Chemical Industries, Zeneca Bio Products, and then Monsanto .The latter company terminated production at the end of 1998 because of high production costs. Biodegradable shampoo bottles, razors, in medical therapeutics and food trays were made out of this material. PHAs can be used to fabricate three-dimensional, porous, biodegradable heart valve scaffold [2], bone fracture fixation [3], manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents [4]. PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing. Poly (3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules synthesized by many gram-positive and Gram negative bacteria. PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as sink for carbon and reducing equivalents. *Ralstonia eutropha* (formerly *Alcaligenes eitrophus*) can accumulate PHAs up to 80 per cent dry weight. More than 80 different forms of PHAs have been detected in bacteria. Only two forms of PHAs, *i.e.*, PHB homopolymer and 3HB-3HV copolymer are commercially produced by Zeneca, United [5]. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellular, especially as their general fitness is not affected. The accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, i.e., when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented [6]. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment [7].

The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low polymer production. The higher production costs, especially raw material costs, make it difficult for PHA plastics to compete with conventional petroleum-based plastics in the commercial market place [8]. Hence, alternative strategies for PHA production are being investigated. PHA production costs could be reduced by several means by using cheaper substrates such as starch and whey [9].

2. MATERIAL AND METHODS

The isolates were grown on waste whey, culture parameters were optimized for the selected efficient strains and metabolic engineering by using acrylic acid as an inhibitor was done to increase the production. The general procedure and special techniques adopted as well as the material used in the study are detailed in this chapter.

2.1 Collection of Samples and isolation of bacteria from Different samples

Samples of solid black colored soil and green colored semisolid soil were collected from stressed condition of effluent treatment plant (unit number 1) of Gokul Dudh Sangh located at Gokul Shirgoan, Kolhapur, Maharashtra, India. Various samples collected were serially diluted and plated on nutrient agar medium. The representative bacterial colonies were picked up, purified and preserved on nutrient agar slants till further use.

2.2.1. Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHB production following the viable colony method of screening using Sudan Black B dye .For rapid screening of PHB producers, nutrient agar medium supplemented with 1 per cent glucose was sterilized by autoclaving at 121^0C for 20 minutes and cooled to 45^0C. The medium was poured into sterile petriplates and allowed for solidification. The plate was divided into 6 equal parts and in each part, a bacterial isolate was spotted. The plates were incubated at 30^0C for 24 hours. Ethanolic solution of (0.02%)

Sudan Black B was spread over the colonies and the plates kept undisturbed for 30 minutes. They are washed with ethanol (96%) to remove the excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production. All the positive isolates were assigned the code numbers based on their source of isolation.

2.2.2 Sudan Black staining method

The isolates were screened for PHB by staining with Sudan black B stain (0.3 in 70% alcohol) [10] and observed under compound microscope .The selected isolates were then identified on the basis of their morphological, cultural, physiological and biochemical characteristics.

2.3 Characterization of PHB Producing Bacterial Isolates

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification.

2.3.1 Morphological tests

The potent PHB accumulating strains SS1 was examined for their colony morphology, pigmentation fluorescence, cell shape and gram reaction as per the standard microbiological procedures [11].

a) Colony characterization

The colony characters *viz.,* shape, color and polysaccharide production were observed on Nutrient agar medium.

b) Gram staining

Twenty four hours old culture was smeared on a clean glass slide and heat fixed. The smear was covered with crystal violet for 30 seconds and washed off with 95 per cent ethyl alcohol. The slide was washed with distilled water and drained. Safranin was applied on smear for 30 seconds as counter stain, washed with distilled water and blot dried. The slide was observed under compound microscope for gram reaction.

2.3.2 Biochemical tests

Biochemical tests were carried out for the identification of PHB with 24 hr old cultures [12].

1) Methyl Red Test: 0.2 ml of bacterial suspension was inoculated into two tubes each containing 2% peptone water. One tube was kept as a control. Both tubes were incubation at 37^0C for 24 hr .Two drop freshly prepared methyl red indicator was added and observed the color change.

2) Voges Proskauer : 0.2 ml of bacterial suspension was inoculated into two tubes each containing 2% peptone water. One tube was kept as a control. Both tubes were incubation at 37^0C for 24 hr. Barritt's reagent i.e. 40% KOH + Napthol.

3) Urease Test: 0.2 ml of bacterial suspension was inoculated into one tubes tube containing urea broth. One tube was kept as a control. Both tubes were incubation at 37^0C for 24 hr.

4) Catalase Test: 0.2 ml of bacterial suspension was inoculated into two tubes. One tube was kept as a control. In another tube hydrogen peroxide was added. Both tubes were incubation at 37^0C for 24 hr.

2.4 Pre-Treatment of Whey Solution

Whey waste was obtained from Warna Dudh Sangh, Warnanagar and Gokul Dudh Sangh, Gokul Shirgaon .To remove excessive proteins in whey solution, the pH of the whey solution was adjusted to 4.5 by the addition of 10 M HCl. The solution was autoclaved at 121^0 C for 15 min and centrifuged at 11 000 rpm in a sterilized bottle for 15 min to remove aggregates. Small protein particles could be removed by filtration using Whatman No. 3 filter paper. The pH of the filtered solution was adjusted to optimum with 12 M NaOH.

2.5 Optimization of Cultural Parameters

Different factors affecting PHB production by the selected promising bacteria was optimized *viz.* pH and temperature.

2.5.1 Effect of pH on PHB production

The bacterial isolates were grown in 100 ml conical flasks containing 50 ml pretreated whey. Different pH of media was maintained *viz.,* 4.0, 5.0, 7.0 and 8.0 and incubated. After 48 h, PHB produced were quantified by crotonic acid assay.

2.5.2 Effect of temperature on PHB production

The bacterial isolates were grown in 100 ml conical flasks containing 50 ml pretreated whey. Different temperature of media were maintained *viz.,*30^0C, 37^0C, 40^0C and incubated. After 48 h,

PHB produced were quantified by crotonic acid assay.

2.5.3 Effect of concentration of Acrylic acid on PHB production

The bacterial isolates were grown in 100 ml conical flasks containing 50 ml pretreated whey. Different concentrations of Acrylic acid were maintained in media *viz*1.5µl/50ml to 12.5µl/50ml for isolated SS strain and 6µl/50 ml to 12.5µl/50ml and incubated. After 48 h, PHB produced were quantified by crotonic acid assay.

2.6 Extraction of Poly-3-hydroxybutyrate

Microorganisms were inoculated pre treated whey Biomass was collected from each media by centrifugation at 4000*g for 15 min at 25^0C then freeze dry the biomass at 4^0C.This powder was taken and 50ml chloroform and 50 ml sodium hypochlorite (4%)were added. Then incubate for 1 hr at 30^0C centrifuge at 4000*g for 10 min. Three separate phases contained non PHB cell material at the top, undisrupted cell at the middle and PHB dissolved in chloroform at the bottom are observed. The upper phase was removed by pipette, middle phase by filtration. Keep the remaining material in open petriplates so that chloroform was evaporated and PHB crystals were obtained. Concentrated 10 ml hot H_2SO_4 was added. The addition of sulfuric acid converts the polymer into crotonic acid which is brown colored. The solution was cooled and the absorbance read at 235 nm against a sulfuric acid blank. By referring to the standard curve, the quantity of PHB produced was determined. Based on the PHB yields, one promising bacterial isolates was selected for further studies.

2.7 Preparation of Standard and Estimation of PHB Production

Standard curve of PHB was prepared [13]. Pure crotonic acid (Himedia) was used to prepare the standard curve. It (200 mg) was dissolved in 10 ml of concentrated H_2SO_4 and heated for 10 min which gave 20 mg/ml of crotonic acid. From the above stock, working standard solution was prepared by diluting 5 ml of the stock (containing 100 mg of crotonic acid) to 10 ml with H_2SO_4, which gives the final concentration of 10 mg/ml (0.01 g/ml). This was used for the preparation of the standard curve. On the basis of standard unknown concentration of PHB was calculated. Crystals of PHB obtained after extraction were converted in to crotonic acid by incubating it in 100^0C boiling water bath for 10 min. Optical density (OD) of the brown color developed was taken at 235 nm on UV-VIS Spectrophotometer.

2.8 Characterization of Poly-3-hydroxybutyrate

White dry crystals were collected and crushed to convert it in to fine powder. Powder was taken to the Common facility center, Shivaji University, Kolhapur. Powder was mixed with Standard Fourier Transform Infrared Spectroscopy (FTIR) grade Potassium Bromide. With the help of Hydraulic Pressure Machine thin tablet was formed. Fourier Transform Infrared Spectroscopy (FTIR) was carried over a range of 200$cm^{-1}$to 4000cm^{-1}.

3. RESULT AND DISCUSSION

Isolation of bacteria from various environmental samples and screened them for accumulation of poly-β-hydroxybutyrate (PHB) within their cells. The efficient isolates were selected and their culture parameters for maximum PHB production were optimized. Different bioeffluents were tested as cheaper substrates for PHB production by the promising isolates. The results obtained during the experimentation are presented in this chapter

3.1 Isolation of Representative Bacteria

As many as 3 samples were collected from Gokul Dudh Sangh, Gokul Shirgao, and Kolhapur. (Liquid, solid and semisolid in nature).Bacteria from theses samples were isolated on nutrient agar amended with glucose (1%). All four bacteria were isolated, purified and maintained on nutrient agar.

3.2 Screening of the Isolates for PHB Production

All the four isolates were subjected for visual screening for PHB production using Sudan black B. The color of the Sudan black B colonies were visually scored in comparison with that of the reference strain .The positive bacteria were assigned the code number depicting the place of their origin as SS, liquid, solid ,SS2 . The isolate

from semisolid condition used for further studies named as Isolate SS

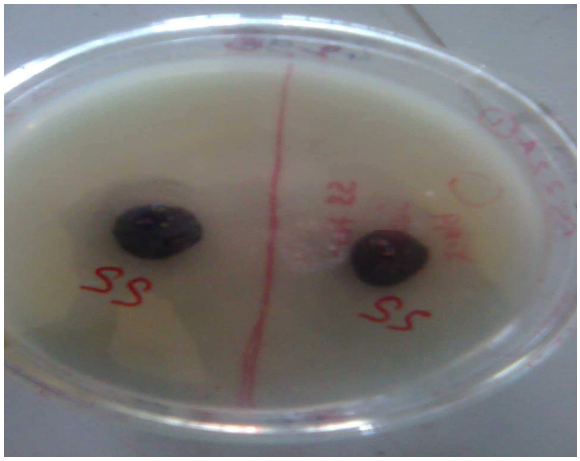

Fig. 1 Blue black colored observed after Sudan black staining of isolate SS 1 on Nutrient Agar

3.4 selection of Promising Bacterial Isolates

Based on the PHB yields, 1 promising isolates was selected. This was isolate SS1 which produced highest PHB yields on whey. The reference strain and isolate have following biochemical characteristics.

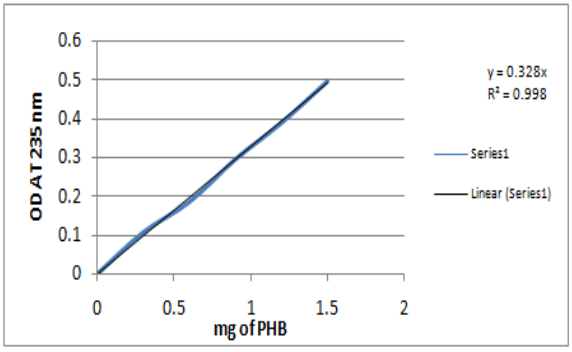

Fig.2 Crotonic acid standard curve

Test	*Bacillus spp. RMG1*	*Isolate SS 1*
Gram Staining	+	+
Citrate	+	+
Methyl Red	+	-
VP Test	+	+
Catalase	-	-

Table 1: Biochemical Test of Bacillus spp.RMGII and Isolate SS 1

4.5 Effect of Different pH Level on PHB Yield

Batch fermentation for different initial pH of media was carried out to analyze effect of pH on yield of PHB. Amount of PHB was estimated after extraction by crotonic acid assay. Data is presented in Fig. 4 .Out of the different initial pH of media tested, pH 4 was found to be optimum for maximum PHB production by isolate SS

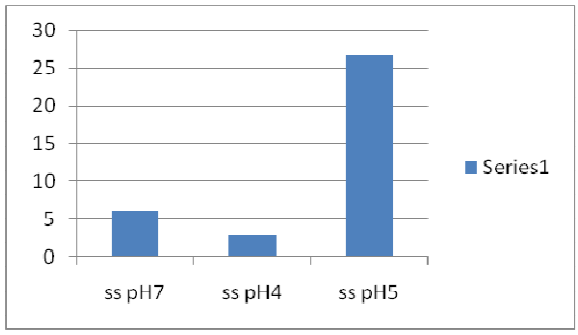

Fig. 3 Effect of pH on %PHB production by Isolate SS1

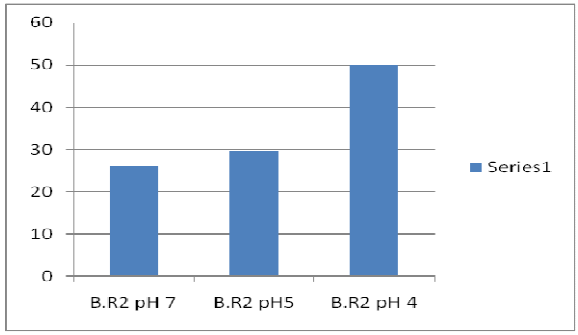

Fig. 4 Effect of pH on %PHB production by *Bacillus spp. RMGII*

4.6 Effect of Different Temperature Levels on PHB Yield

Different temperatures were maintained in the media prepared. Their effects on PHB production were evaluated. Data is presented in Fig. 4 Out of the different temperatures of media tested, 37^0C was found to be optimum for maximum PHB production by isolate SS and reference strain *Bacillus spp. RMG II* . At 37^0 C the highest PHB of 47 % was produced by which was significantly higher than PHB produce at other temperature.

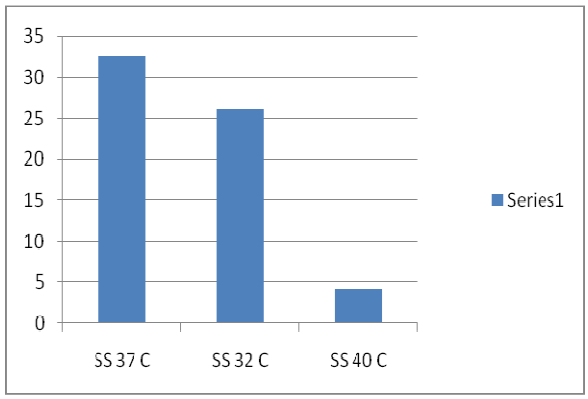

Fig.5 Effect of Temperature on % PHB production by isolate SS1

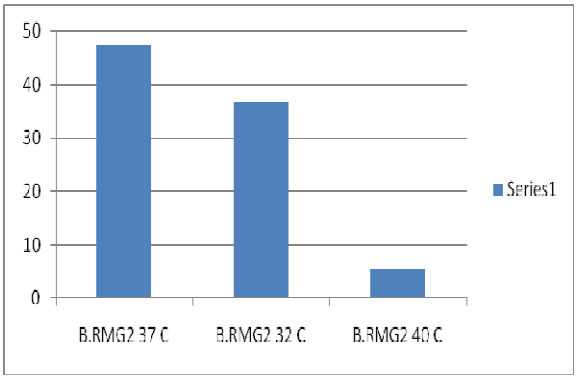

Fig.6 Effect of temperature on % PHB production by Bacillus spp. RMGII

4.7 Effect of Different Concentration of Acrylic Acid Levels on PHB Yield

Different concentrations of acrylic acid were maintained in the media prepared. Their effects on PHB production were evaluated. Data is presented in Fig. 5 Out of the different concentrations of acrylic acid of media tested 2.2µl/50ml and 7.7µl/50ml was found to be optimum for maximum PHB production by isolate SS and reference strain *Bacillus spp RMG* respectively. At 37^0 the highest PHB of 57.73% was produced by which was significantly higher than PHB produce at other concentration acrylic acid.

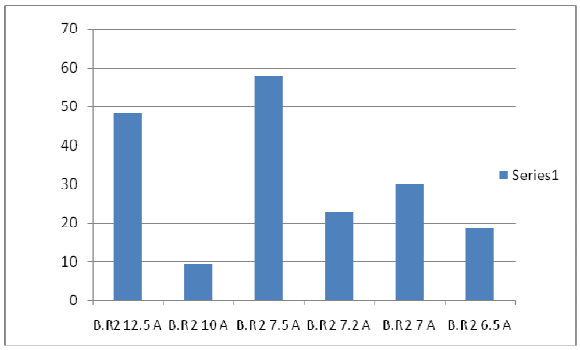

Fig.7 Effect acrylic acid on production of PHB by *Bacillus spp. RMG1*.

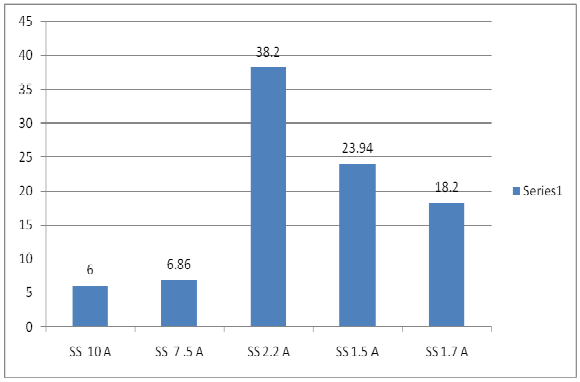

Fig.8 Effect acrylic acid on production of PHB by isolate SS1

4.8 PHB Production at optimized pH, Temperature and Acrylic Acid Concentration

At 37^0C, pH 4 and 7.5µl/50ml concentration of acrylic acid maximum production of 51.95% by *Bacillus spp.RMG* and at same temperature and pH and 2.2µl/50 ml concentration of acrylic acid, 30.91% PHB production was observed by isolated *SS* strain.

4.9 Fourier Transform Infrared Spectroscopy (FTIR) Studies of poly hydroxybutyrate

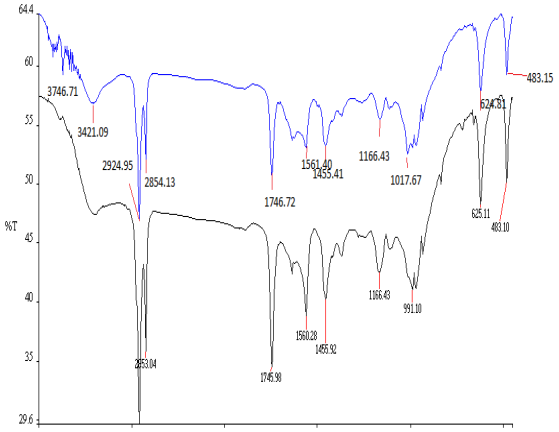

Fig. 9 FTIR of studies isolated SS 1 (blue) and *Bacillus spp. RMG2* strain (black)

1. 625.11 and 624.81, 483.10 and 483.15 - C-H rocking,
2. 991.10, 1017.67 and 1166.43 - C-O Stretching,
3. 1455.92 and 1455.41 - C-H of CH_3,
4. 1561.40 and 1560.28 -C=O Stretching,
5. 2953.04 and 2854.13 - C-H stretching,
6. 1455.92 and 1455.41-C-H of CH_3,
7. 3746.71 – O-H – Impurities.

Peak in both samples at 2953 cm^{-1} and 2924.95 cm^{-1} shows methyl group with its probable position in basic structure as R group indicates the given sample is Poly (3-hydroxybutyrate) while rest of the peak indicate the functional group forming basic framework of Poly hydroxyl alkanoate(PHA).

4. DISCUSSION

Economics of production of bioplastics mainly affects on feasibility for its adequate use to fulfill the actual needs in day today life. Cost of media used in the production play a critical role in overall economics. Whey waste of dairy industry provide ample amount of carbohydrates

and proteins as well as lipids and fats. Production of PHB observed on whey waste sample was approximately 0.12 gm per gm of biomass. Acrylic acid blocks the beta oxidation of fatty acid degradation. Accumulation of fatty acid in larger amounts inside the cell results in to the production of polyhydroxybutyrate in superior amounts due to the diversion of intermediates towards the polyhydroxybutyrate production. As the isolate were obtained from nutrient deficient and stress condition they are having better ability to accumulate the nutrients during its stationary phase or starvation conditions for its survival. When these isolates are grown in an optimum condition with suitable nutrients gives good polyhydroxybutyrate production. Acrylic acid concentration above the optimum level is inhibitory for its growth so it is optimized on a range of 10µl/lit to 250µl/lit. Its optimum concentration gives sufficient growth and maximum polyhydroxybutyrate production. Physiological conditions such as pH, temperature also affect the fermentation processes that are optimized for maximum production of polyhydroxybutyrate. It should be noted that organisms which are isolated from stress condition and capable of tolerating the acid concentration gives superior production of polyhydroxybutyrate by mentioned strategy.

5. ACKNOWLEDGMENT

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