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REVIEW

Microbial Production of Biopolymer Polyhydroxybutyrate (PHB): Current Challenges and its Application

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Plastics are suitable for many industrial applications as they are inexpensive, sturdy, resistant to deterioration and highly versatile. As advantageous as plastics made from petroleum are, it remains the most major topic of concern among environmentalists and the scientists. Polyhydroxyalkanoates are biodegradable polyesters produced by a wide range of microbes as a source of energy for metabolism, carbon, and oxidoreduction; since they exhibit some properties, which are remarkably similar to those of polyolefins derived from fossil fuels. The literature review conducted over the past decade reveals that polyhydroxybutyrate (PHB) has the potential physical qualities for food packaging material, single-use plastic goods and medicinal sectors. Polyhydroxybutyrate (PHB) is a bacterial biopolymer that has begun to replace petrochemical plastics due to the present trend towards the efficient utilisation of relatively cheaper substrates for its production. This review discusses explicitly the developments in the analysis of microbial biopolymer PHB and additionally provides an outline of current industrial applications, Production, Extraction methods, genes involved, microbes that are capable of producing PHB, challenges faced during production and degradation of PHB.

Keywords: Polyhydroxybutyrate, Biopolymer, Inexpensive substrates, Industrial applications, Degradation.

INTRODUCTION

Biopolymers, often known as "biodegradable polymers," are polymeric materials that can degrade through the enzymatic action of microorganisms including bacteria, fungi and algae in a habitable environment. Lipids, proteins and carbohydrates are the most crucial elements of biobased products including bioplastics, biopolymers and biobased polyurethane, which microalgae and some cyanobacteria species are capable of producing in significant amounts [1]. A number of disciplines, including the manufacture of bioplastics, have been enhanced by microalgal biomass in recent years [2]. There are some species of microorganisms that use polyhydroxyalkanoates (PHAs) for carbon storage and reducing equivalents as part of their metabolism which have physical properties equivalent to that of petrochemical ones [3]. More than 90 bacterial genera and a few haloarchaea species have currently been identified as major PHA producers. Polyhydroxyalkanoates (PHAs) are completely biocompatible, biodegradable, resistant to UV irradiation, insoluble in water, tunable in their thermal behaviour and open to a variety of functionalization [4]. Polyhydroxyalkanoates (PHAs) are made by microbes using sugar and/or lipids as carbon sources whereas polyesters are made by microbes using hydroxyalkanoates as a carbon source. Due to their similar properties, PHAs can be used as alternatives to petrochemical-based plastics like polypropylene in plastic bags and containers [5]. Polyhydroxyalkanoates (PHAs) biosynthesis is facilitated by an extremely interconnected network of enzymes, including PHA synthase, acetoacetyl-CoA-reductase, acetyl-CoA-acetyl-transferase and a gene cluster containing phaZ, phaM, phaC, *etc.* [6]. The bioplastics sector has been developing rapidly across the globe in the last few decades. This review focuses on the current technology used for the production of bacterial biopolymers and their application in various sectors. Microbes produce polyhydroxybutyrate (PHB) through the condensation of two acetyl-CoA molecules, which leads to the formation of acetoacetyl-CoA and the subsequent condensation of hydroxy-butyryl-CoA. This final complex, consisting of a short-chain

length polymer derived from PHA (3-4 carbon atoms in length), would be used as a single component in the synthesis of PHB [7].

Currently, biodegradable plastics altogether, including Polylactic acid (PLA), PHA, starch blends and others, account for more than 51% (over 1.1 million tonnes) of the global bioplastics production capacities. The production of biodegradable plastics is expected to increase to over 3.5 million in 2027 due to a strong development of polymers, such as polylactic acids (PLAs) and polyhydroxyalkanoates (PHAs). The market for PHAs is expected to rise significantly between 2022 and 2029. According to Data Bridge Market Research's analysis, the market is estimated to reach USD 144,530.99 million by 2029 and will grow at a CAGR of 5.4% from 2022 to 2029 [8].

Bisphenol A (BPA) is a chemical molecule found in plastics because of its potential to cause harm to human health, especially through interference with hormone regulation and has drawn special attention. Research reports that when plastic products are used to store acidic or basic products or even use for microwaving they tend to release BPA. In the United States, measurable levels of BPA have been detected in the urine of majority of people. It is further detected in blood, saliva, breast milk and amniotic fluid also [9].

The male reproductive system is particularly vulnerable to the harmful effects of BPA, which include infertility, impotence, epididymal damage, testicular abnormalities, hormonal imbalance and sperm failure. As according to European Bioplastic & Nova Institute (EBNI); a non-renewable fossil hydrocarbons are made up of 99% plastic (petroleum and natural gas). Market projections suggest that plastic production will double in the next decade to 348 million tonnes to meet market demand [10]. Petroleum-based plastics contribute significantly to global warming gases during their entire lifespans. By 2050, the increasing demand for plastic materials will cause an increase in worldwide emissions of up to 6.850 Gt of CO_2 equivalent [11]. According to Geneva environment network, which published a research report in 2023, has shown that microplastics can be harmful to our health and serve as pathways for infections to infiltrate our systems, which accelerates the spread of disease [12]. The rapid depletion of natural resources and the public's rising awareness of plastic pollution have been the main driving forces behind the production of biodegradable polymers with biological origins in recent years [13]. To overcome this drawback of non-degradability, alternatives are preferred to reduce the rate of pollution. There are various types of bioplastics available in the market in the form of starch-based, PLA, PHB, *etc.* in recent years, biopolymers are on trend to be used as an alternative for commercial petrochemical plastics.

Polyhydroxybutyrate (PHB), which has tensile and thermoplastic qualities comparable to polypropylene and polyethylene, is one of the most promising alternatives to petroleum-based plastics. Due to its renewability, biodegradability and biocompatibility, PHB has been utilized in tissue engineering, surgical devices and medication delivery, in addition to its commercial applications as an eco-friendly and more sustainable alternative to conventional plastics [14]. When compared to plastics made from petrochemicals, PHBs have a higher production cost, which is a significant issue for their manufacture. As a result, substantial work has been done to use inexpensive carbon sources to produce PHBs at a lower cost [15]. This review will give detailed knowledge about PHB, its value and techniques to improve its production in a cost-effective manner.

Role of PHB in microbial metabolism: On exposure to heat shock and reactive oxygen species, 3-hydroxybutyrate and its oligomers exhibit a protective effect against protein aggregation and cellular damage (Fig. 1) [16,17]. Polyhydroxyalkanoates (PHAs) accumulation is a typical metabolic mechanism used by many bacteria to deal with cold conditions and other adversities, as evidenced by the isolation of several PHA-producing bacterial strains from Antarctic freshwater and soil [18]. PHB enables easier adoption, distribution and replication in any media [19]. The presence of PHB preserves biological molecules including RNA and proteins during a famine and crucial to the process of sporulation. They function as a sink for minimizing power and serve as a carbon and energy storehouse. Research investigation of polyhydroxybutyrate (PHB) accumulation suggested that it contains defensive mechanisms when bacteria are exposed to thawing and freezing conditions [20].

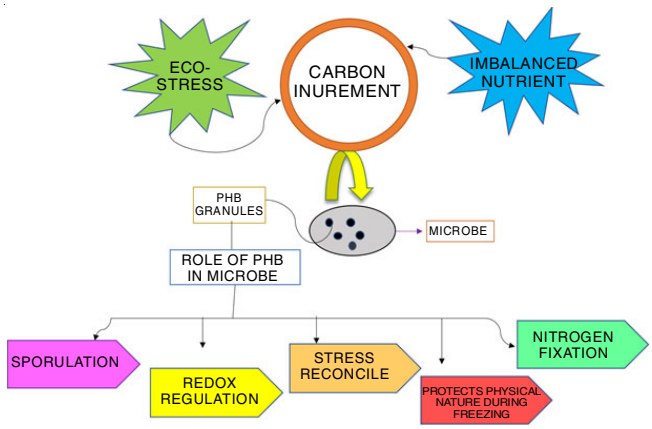

Fig. 1. Role of PHB in microbial metabolism [16,17]

Properties of PHB biopolymer: PHB has a multitude of general characteristics that make it potentially suitable as a fabric for clothing in the future. This includes it being water insoluble, moderately resistant to hydrolytic degradation, having good oxygen permeability, resistant to UV, susceptible to acids and bases, soluble in chlorinated hydrocarbons, including chloroform, non-toxic, biocompatible, having high tensile strength (40 MPa), sinking in water to facilitate anaerobic biodegradation and less sticky when melted. Using PHA synthase, PHA polymers with variable subunit compositions and consequently different physical and thermal properties can be manufactured to order for particular purposes [21]. The high melting temperature is relied on by strong intermolecular interaction, which is responsible for the slight disparity between these two temperatures. Similar to how conventional polymers function, polymerization with co-monomers can solve these issues with pure PHB [22]. The most purest form of PHB, a thermoplastic, is brittle and known for its great rigidity, crystallinity and relative brittleness. It has a tensile strength of 40 MP which is almost equal to that of synthetic plastic polypropylene. PHB

sinks in water whereas synthetic plastic stays afloat on the surface. However, the sinking of PHB smooths the path of its anaerobic biodegradation in sediments. It possesses a transition temperature (T_g) of 5 ºC and a high mass. In addition, PHB has a T_m = 175 ºC freezing point, which is only slightly lower than the temperature at which it starts degrading to crotonic acid (185 ºC), which makes processing time-consuming [23]. The ability of microbes to accumulate polyhydroxy butyrate (PHB), which is well-recognized and distinguished as polyhydroxyalkanoate, is typically employed as a taxonomic trait related to its durability [24]. PHB serves as an energy source for sporulation in *Bacillus* sp. and the oxidizing substrate for the protection of metabolic processes in *Azotobacter* sp., in addition, to serving as an energy reserve material [25]. Following Sudan black-B or Nile blue staining procedures, these inclusion bodies, which are confined to the cell's cytoplasm, can be observed under a microscope. Under nitrogen starvation, it was discovered that the PHB granule count stayed consistent at 8-12 throughout cell development. The outflow of these important substances from the cell is prevented by the polymerization of soluble intermediates to insoluble molecules, which stops the cell from experiencing changes in its diffusion state [26]. PHB is particularly crystalline because of its linear chain structure, which includes both the amorphous and crystalline phases. It can be discovered as a virgin polymer, in copolymers and combinations. It is created as a carbon source in a huge diversity of generating bacterial strains and processed industrially through bacterial fermentation. PHB is superior to the synthetic polymers in several ways for the manufacture of specific packaging applications. Polypropylene (PP) and polyethylene (PE) are both outperformed by PHB barrier permeability and PHB is also shown to be less stiff and more flexible. Additionally, when compared to polyethylene terephthalate (PET) and polyvinyl chloride (PVC), where PHB demonstrates the smart barrier qualities [27].

Interpretation of PHB production by different microorganisms

Array of bacterial community that produce PHB: Soni *et al*. [28] produced PHB in a cost-effective way using banana peel and mustard cake for production attained 2.11 g/L under optimized conditions. Bacteria that generate PHB were isolated and 16S rDNA sequencing identified *Sphingomonas* sp. as the most prolific PHA producer. The strain's capacity to accumulate PHA was examined after being cultured on utterly unique sugars and organic acids. After maturing on a few organic acids, sugar alcohols, disaccharides and aldohexose, the strain may acquire PHA but not starch, pentoses or ketoses. With sucrose or mannose among the sugars investigated, a tributary to 55-60% of cell biomass, a high PHB output was observed. From natural habitats, nitrogen-fixing bacteria, aerobic and non-parasitic were found [29]. According to the results of screening those isolates, almost 70% of *Azotobacter* sp., isolates are comparatively capable of accumulating PHB. While the majority of strains had PHB levels between 25 and 47% of cell dry weight, approximately 7 isolates accumulated PHB equivalent to more than 50% of their cell dry weight. When cultivated under ideal conditions, one of the *Azotobacter* sp., strains have been demonstrated to synthesize the polymer for around 70% of the cell dry mass [25]. Grass field soil included Gram-positive bacteria that produced polyhydroxyalkanoate (PHA). Because strain INT005's PHA productivities were higher between 37 and 45 ºC compared to that of *Bacillus megaterium* and *Ralstonia eutropha*, the PHA exhibited a significant thermostability. Non-thiobacteria that are phototropic and purple sulfur bacteria can synthesize PHB [30]. When cultivated on octinoxate, a strain of *Pseudomonas* sp. that was isolated from the Antarctic regions produced significant amounts of polyhydroxy butyrate (PHB). This isolate was recognised by its distinct behaviour patterns and the sequencing of its 16s ribosomal RNA gene [31]. The PHB-producing bacteria have been found in a wide range of environments, including tannery effluents, sewage sludge, field soil and garden soil. In comparison to other sources, they obtained more PHB-positive strains from tannery effluents and waste product sludge [32]. Acquired *Bacillus* strains from Ankara, Turkey's grassland soils and they were classified as *Bacillus sphaericus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus circulans, Bacillus brevis*, *Bacillus coagulans* and *Bacillus licheniformis*. Polyhydroxy butyrate (PHB) production of different strains was determined using the spectrophotometric technique and showed that PHB synthesis varied depending on the cell dry weight from 1.065 to 41.09% (w/v). The highest PHB production and efficiency ratio were found in *Bacillus brevis* M6 (41.09%) [33].

PHB producing cyanobacteria from diverse origin: Rueda & Garcia [34] undergone the scale batch fermentation *Synechocystis* sp. produced a maximum quantity of 5.1 mg PHB L^{-1} d^{-1}. One of the studies discusses how *Nostoc muscorum* can build up PHB under nitrogen-limited chemo heterotrophy and mixotrophic conditions five times more efficiently than most other organisms can synthesize under photoautotrophic circumstances. The capacity of cyanobacteria to produce PHB using solar energy can save costs and CO_2 emissions [35]. About 23 cyanobacterial strains have characterized for PHB production and the highest concentration was detected in *Nostoc muscorum* NCCU-442 and the lowest was found in *Spirulina platensis* NCCU-S5 whereas *Cylindrospermum* sp., *Oscillatoria* sp. and *Plectonema* sp. doesn't produce PHB [36]. The ability of cyanobacteria to produce PHA using CO_2 and sunlight as both carbon and energy sources is also well recognized. The PHA synthase enzyme is found in these naturally occurring photosynthetic bacteria that generate oxygen. However, most of the cyanobacteria have been found to possess PHB homopolymer are known. Both *Synechocystis* sp., PCC6803 and *Spirulinapla tensis* UMACC 161 [37] are cyanobacteria that may synthesize PHA and accumulate PHB up to a maximum of 12% of dry cell weight. When there is insufficient phosphate available, *Nostoc muscorum* and *Synechococcus* sp. MA19 has produced PHB [38]. The PHB-producing cyanobacteria *Synechococcus leopoliensis* was cultivated in an open thin-layer photobioreactor grown in mineral *Z*-medium and produced 0.7 g L^{-1} d^{-1} of PHB [39]. When PHB from Spirulina LEB 18 was added to nanofibers, conductance, breakage, tensile strength, flexibility and dilation were gradually increased and became

more refined [40]. Overall, it was discovered that spirulina PHB nanofibers performed better in terms of mechanical qualities than commercial PHB nanofibers. It was shown that the diatom *Phaeodactylum tricornutum* could produce PHB at a rate of up to 11.1% dry weight of its biomass when exposed to the bacterial PHB pathway *Ralstonia eutropha* U16 [41]. In addition to PHB, the macroalgal polysaccharides were commonly used as blending ingredients. Despite the presence of a significant amount of siliceous material, algal fiber was mixed effectively with poly-ε-caprolactone (PCL) and poly-4-hydroxybutyrate (PHB). When linked at the appropriate concentration, macroalgal polysaccharides were synthesized from *Ulva armoricana*, which demonstrated potential utility in PHB polymers as a filler. Additionally, thermal characterization demonstrated that blends may be processed at high temperatures without obviously complicating things [42].

Production of PHB by indigenous actinomycetes: As observed by Trakunjae *et al*. [43], a rare actinomycete species *Rhodococcus pyridinivorans* BSRT1-1 produced 43.1 ± 0.5 wt.% of dry cell weight (DCW) of PHB biopolymer. The PHB generated by actinomycetes can also be used in place of synthetic polymers in a wide range of commercial applications. Actinomycetes were understood only by a few reports on their application in the synthesis of PHB. The adaptable microorganisms known as *Streptomyces* sp., can produce both primary and secondary metabolites, including antibiotics. The ability of *Streptomyces* sp., to synthesize and generate PHB is still under development [43]. *Streptomyces incanus* BK128 isolated from the rhizosphere soil sample used for the production of PHB [44]. The presence of poly-3-hydroxybutyrate (PHB) in 10 distinct strains of the genus *Streptomyces* sp. was examined. All of the halophilic bacteria produced PHB, as shown by gas chromatographic analysis, with the maximum accumulation falling between 1.82 to 12.08% in dry cell weight. The PHB was identified using FT-IR spectroscopy from *Streptomyces coelicolor*. Research on the relationship between PHB use and production in *Streptomyces coelicolor* suggests that PHB may be utilized as a carbon storage molecule during the synthesis of antibiotics [40]. Rare actinomycetes *Aquabacterium* sp., A7-Y produced 10.2 g/L of PHB in 5 L fed-batch fermenter after optimization [45]. Streptomyces incanus BK 128 which was isolated from the Rhizosphere of eggplant by Rezk *et al*. [46] used for production of PHB biopolymer using different agricultural waste synthesized maximum quantity of 2.82 g L^{-1}.

PHB synthesis by yeast cells: To understand more about PHB synthesis in eukaryotes, yeast cells were utilized as models. PHB production was maximized to 1.8 g L^{-1} by using *Pichia kudriavzevii* TSLS24 yeast, which was isolated from sediment samples during Vietnam's livestock waste treatment [47]. The programming of novel pathways appears to be a useful alternative to the manufacture of PHBs. Yeasts have several advantages over bacteria when it comes to the synthesis of PHB. The Food and Drug Administration has also recognized yeast species such as *Candida utilis*, *Klyveromyces maximus* and *Saccharomyces cerevisiae*, production of PHB biopolymer in two transgenic yeasts. One of these, *Saccharomyces cerevisiae*, had PHB synthase genes from *Ralstonia eutropha* inserted into its chromosome, but not the other, *Schizosaccharomyces pombe* [18]. Poly(D-lactic acid) and copolymer PHB were produced and characterized in the yeast saccharomyces cerevisiae by Ylinen *et al*. [48] resulting in the synthesis of 11% PHB of the total dry cell weight. The highest P(LA-3HB) accumulation 3.6% of CDW. Out of 32 strains tested in whey for PHB formation, only *Candida tropicalis* was identified by Zisha *et al*. [49]. The cost-effective different carbon sources analyzed for PHB production is shown in Table-1.

TABLE-1
COST-EFFECTIVE CARBON SOURCES ANALYZED FOR PHB PRODUCTION

PHB positive strains	Substrate used	Ref.
Bacillus badius MTCC 13004	Banana peel and mustard cake.	[28]
Candida tropicalis	Whey	[49]
Bacillus cepacia	Spent coffee grounds	[50]
Cupriavidus necator H16	Waste cooking oil	[51]
Bacillus wiedmannii AS-02 OK576278	Fruit Peel Waste	[52]
Pseudomonas aeruginosa	Poultry waste	[53]
Ralstonia eutropha Re2058/pCB113	Animal by-products	[54]
Pseudomonas canadensis	Tapioca powder	[55]
Lysinibacillus sp.	Sugarcane bagasse	[56]
Escherichia coli	Algae biomass residue	[57]
Cupriavidus necator	Sugarcane molasses and vinasse	[58]
Azohydromonas australica DSM 1124	Whey	[59]
Streptomyces incanus BK 128	Wheat bran, rice bran, rice straw and molasses	[47]
Cupriavidus necator	Chicken feather hydrolysate-nitrogen source & waste frying oils-carbon source	[60]
Burkholderia cepacian USM	Rice husk	[61]
Bacillus sphaericus NCIM5149	Wheat bran	[62]
Bacillus megaterium R11.	A bunch of empty oil palm fruits	[63]
Bacillus subtilis NG220	Waste from the sugar industry	[64]
Pseudomonas stutzeri	Whey	[65]
Pseudomonas aeruginosa	Sugar cane molasses	[66]
Cupriavidus necator	Waste rapeseed oil	[67]
Pseudomonas guezennei	Oil of koprah	[68]
Pseudomonas putida CA-3	Chemically synthesized plastic debris	[69]
Alcaligenes latus	Cane molasses, maple sap. Beet molasses.	[70]

Screening methods for PHB-producing organisms and characterization of PHB: Distinctive PHA granules can be stained using Nile blue A, Sudan black B and Nile red. Nile blue A specifically stains PHAs, where its presence is recognized by intense orange fluorescence when observed under a UV-transilluminator [71]. The PHA granules' size, number, physico-chemical characteristics, compound composition, as well as their structure, differ depending on the organism. A scanning electron microscope observation of PHB granules isolated from *Bacillus thuringiensis* cells has revealed a persistent spherical form with a mean diameter of 5 µ [72]. Simple staining techniques were used to find PHB in marine microbial sources. Sudan Black staining in addition to Nile blue is one of the several phenotypic detection techniques used to identify intracellular PHA granules in PHA producers, staining that produces granules of dark blue or luminous colour. The process of screening a large number of environmental isolates requires a lot of time and tedious procedure, even though these approaches are extremely sensitive [73]. Plate assay with Sudan black B staining is the most basic method for detecting PHB production. The cultures were incubated for 24-48 h before the stain was put over the plate and kept for 30 min at room temperature. The presence of deep greenish blue indicates that the isolate is positive for PHB synthesis [74]. Using PHB selective media, with the following components: 2.5 g H_2KO_4P, 25 g HNa_2O_4P, 100 g mannitol, 20 g NaCl, 1 g $MgSO_4$, 100 g $C_3H_3NaO_3$, 10 g peptone, 1.2 g bromothymol blue and 20 g agar for 1000 mL distilled water, can also be used for secondary screening of PHB accumulation. The test isolates' ability to synthesize PHB was validated by the appearance of bluish colonies on the culture-inoculated plates after 3 days of incubation at 35 ºC [75].

The isolates were plated over sterile Nile red media after mineral salt media had been added with 0.5 g of Nile red per mL of medium after incubation (48 h) when exposed to ultraviolet light (312 nm) to check for the buildup of PHB, colonies with pinkish colour showed PHB production and were considered a positive result [76,77]. According to GC-MS spectra of the PHB collected by various isolates and PHB standards, the molecule produced is specifically PHB, except VK-9, which contains an extra peak at 5.27 [78]. Atomic force microscopy (AFM) and transmission electron microscopy (TEM) have demonstrated the emergence of early granules in *Wausteria eutropha* H16. The development of PHB granules was the subject of electron microscopy investigations in *Chlorogloea fristschii* and *Bacillus megaterium*, but there has been no study on *Alcaligenes* sp., intracellular PHB granule synthesis that simultaneously evaluates its quantitative and qualitative characteristics [79]. PHAs are identified with a phase-contrast light microscope as distinct granules with a diameter of between 0.2 and 0.5 µm that are confined to the cytoplasm of cell. It appears as an electron-dense entity under a transmission electron microscope (TEM). PHA will have a mass of between 2×10^5 and 3×10^6 Daltons [80]. A scanning electron microscope observation of PHB granules isolated from *Bacillus thuringiensis* cells has revealed a persistent spherical form with a mean diameter of 5 microns. The polymer was recognized as an isotactic homopolymer by nuclear magnetic resonance spectra. Three groupings of PHB homopolymer signal characteristics were visible in the spectrum. The methylene radical adjacent to an asymmetric carbon atom bearing a single proton was responsible for the quadrant doublet at 2.61 ppm and the methylene group was responsible for the multiplet at 5.30 ppm. At 7.25 ppm, chloroform is emitted as a chemical shift indicator. The alkyl group connected to one proton was associated with the doublet at 1.3 ppm. FTIR research revealed two absorption bands at 1280 cm^{-1} and 1735 cm^{-1}, which are recognized by the C=O and C-O stretching groups. The polydispersity index of the polymer was isolated from *Bacillus thuringiensis* R1 cells and shown by the gel permeation chromatography (GPC) study [72, 73].

Factors influencing PHB synthesis: The most crucial element to consider while producing PHB is the carbon/nitrogen (C/N) ratio because it has been discovered that a C/N ratio of 25% produced significantly more PHB. The major disadvantage is the high cost of producing PHB, particularly the expense of carbon sources. The C/N ratio is yet another important aspect that affects how PHB is synthesized, along with carbon. A high C/N ratio in media promotes the accumulation of PHB. Therefore, a good, unrefined and affordable carbon source must be used to produce PHB [74]. In addition, pH is a very significant aspect of the formation of PHB. Its production increases under nutrient-limited conditions, whereas biomass growth increases under situations of high nitrogen availability but with little PHB production. Reduced phosphorus and nitrogen concentrations favour PHB buildup rather than complete formation under the given circumstances. Utilizing different acids as carbon sources have resulted in improved PHA synthesis. In phosphate deficient conditions, *Aulosira fertilissima* may produce 51.9% and 76.3% of PHB with the addition of 0.5% each of citrate and acetate, accompanied by 5 days of dark incubation [81]. By looking at totally distinct studies, it has been determined that the increase in cell mass is directly related to the increase in PHB accumulation. The majority of PHB must be stored for a long time at a high substrate concentration. Faster PHB generation is caused by the medium's lower organic content. Because stored PHB is much greater at higher substrate concentration rates after 40.31% of cell dry weight, PHA production conception is dependent on substrate concentration. A study examining the impact of pH on fermentation medium has concluded that more PHB will be produced with an initial alkaline pH of 9. But in contrast to basic pH 9 (8.5%) and acidic pH 6, neutral interaction circumstances (pH 7) lead to PHB production that has reached up to 25% of the dry weight of cell (15%) [38, 39].

Mechanism of PHB biosynthesis: Among the various PHA components, PHB is the one that has received the most attention and study thus far. Acetyl-coenzyme-A (acetyl-CoA), produced metabolically by bacteria, is transformed into PHB by the combined action of three biosynthetic enzymes. The Three-step process of PHB biosynthesis is shown in Fig. 2.

During typical growth, free CoA prevents 3-ketothiolase from exiting the TCA cycle. However, during non-carbon dietary restrictions, which limit acetyl-CoA entry into the Krebs cycle, the excess is diverted toward PHB biogenesis [82]. The bio-

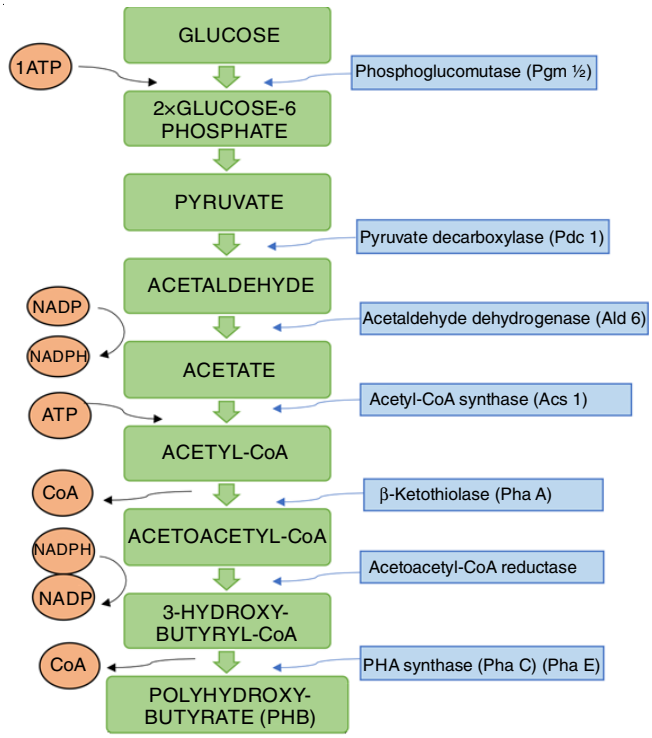

Fig. 2. Schematic representation of PHB biosynthesis pathway

synthesis of bacterial PHB may involve a series of catalyzed chemical reactions that change the initial carbon source, typically a sugar, into the PHB biopolymer. Three key enzymes are involved *viz*. the acetoacetyl-CoA enzyme that converts acetoacetyl-CoA into -ketothiolase, the enzyme that converts acetyl-CoA into (*R*)-3-hydroxybutyrate-CoA (PhaB) [83]. The amount of acetyl-CoA will rise while the level of CoA falls under unbalanced growth conditions. This initiates the three-step process for the creation of PHB by the enzyme α-ketothiolase. The main enzyme, α-ketothiolase, is suppressed when the amount of Co-A rises and the biopolymer's synthesis is also suspended. The enzyme α-ketothiolase, which may serve as a crucial catalyst for the synthesis, controls this activation and deactivation mechanism of the PHB synthesis [84].

It is well-known that several different bacterial species can build up PHB. PHA has been found in a variety of habitats, including mangrove environments, ponds, sewage sludge, soil, gas field soil and marine sediments [30,85]. PHA-producing microorganisms are studied in two aspects: The underlying genetic makeup of the organism from a microbiological perspective and the economics of PHA production.

Nicotinamide nucleotides do not oxidize when oxygen is insufficient. The TCA cycle's efficiency may be impacted as a result, as well as the activity of the NADH-reduced enzymes citrate synthase and isocitrate dehydrogenase. As a result, acetyl-CoA builds up and the intracellular concentration of free CoA occasionally decreases. The inhibition of β-ketothiolases is reduced to some extent and PHB production is promoted by an increase in the acetyl-CoA/CoASH ratio. PHA is built up during phosphate and magnesium deficiency from the earlier stage of cell formation. Phosphorus is one of the vital nutrient sources for living organisms and is necessary for the control of physiological conditions and energy metabolism. Magnesium ions are known to accelerate growth. Low phosphorus levels would prevent high mass and PHB content from being achieved. Phosphate does not directly contribute to protein composition, hence phosphate restriction can support persistent cell development [86]. In the recycling of energy intermediates, phosphorus is involved. When ATP and ADP are not present, NADP is needed, which allows for the synthesis of PHA. Acetyl-CoA and NAD (P) H are constrained if nitrogen is limited. NAD (P) H that has been liberated cannot be used by reductive synthase, which makes amino acids. Acetyl-CoA will be directed toward PHB synthesis since there is a corresponding lack of protein synthesis. The effective synthesis of PHA requires the presence of acetyl-CoA and NADPH. The intracellular accumulation of acetyl-CoA should be more than usual. It has been demonstrated that the flow of acetyl CoA into the PHB biogenesis route for energy storage or the TCA cycle for cell development depends on the quantitative relationship between NADPH and NADP. Increased NADPH levels significantly improve PHB accumulation [87].

Contemporary approaches for PHB extraction: Mongili *et al*. [88] utilized dimethyl carbonate for the extraction of PHB which showed 73% of yield when using wet pellets when compared to chloroform-based extraction method. In an effort to reduce the use of solvents and chemicals. Murugan *et al*. [37] reported the biological extraction of polymer using the intestines of mealworms. It is possible to employ this method for profitability. Mealworms may quickly consume the freeze-dried cells of *Cupriavidus necator* and observed that PHA-containing white fecal pellets were expelled. Aramvash *et al*. [89] have undergone the extraction of PHB from *Cupriavidus necator* using eight different solvents, which showed ethylene carbonate to be a good solvent for extraction when compared with standard methods. A recovery yield of 98.6% and purity of 98%, have been obtained. The PHB polymer can be further refined in an environment friendly manner by pretreating it with sodium hydroxide, water or low concentrations of surfactants like sodium dodecyl benzenesulfonate and sodium dodecyl sulfate rather than chloroform and other dangerous solvents, according to a study by Saharan *et al*. [38]. Interestingly, the PHB that is physiologically recovered had a similar molecular weight (Mw) to the PHB obtained by chloroform extraction. This shows that PHB granules' molecular weight is not compromised by the biological extraction procedure, which is acclaimed as the most efficient extraction method. After being washed in water, biologically derived PHA has a purity of about 90%. A patent have been filed for the method in which PHB granules were partially purified using Sprague Dawley rats. The rats received just freeze-dried *C. necator* H16 cells for 7, 14 and 28 days. There had never been a fatality during the entire study. Kunasundari *et al*. [90] stated that PHA is intracellularly stored as granules in the cytoplasm of bacteria and must be extracted by breaking the cell wall. So the extraction procedure also entails aqueous two-phase systems, flotation techniques, chemical digestion, disruption of supercritical fluids, gamma irradiation, enzymatic treatment, detergents and disruption of bead mills. According to Kunasundari *et al*. [90], the PHB is

often extracted from recombinant *E. coli* using the chemical digestion approach. At 33 ºC, sodium hypochlorite is applied to the biomass for 1 h. However, the purification process has a number of downsides, including severe PHB molecule disintegration, significant wastewater generation and the need for processing to remove the surfactant from effluent. Tao *et al.* [91] reported a novel and environmentally friendly solvent acetone/ethanol/propylene carbonate in the ratio of 1:1:1 for PHB. It showed 92% purity of PHB with 85% yield from dry biomass and 90% purity from wet biomass with 83% of PHB yield. Aramvash *et al.* [89] also reported the extraction using two different solvents butyl acetate and ethyl acetate, which when compared butyl acetate had a higher recovery percent of 96% and PHB purity of up to 99% than ethyl acetate where both are compared to standard chloroform. PHB was isolated from *Cupriavidus necator* by Fiorese *et al.* [92] using 1,2-propylene carbonate, with a yield of 95% and a purity of 84%.

Pathway of PHB biopolymer degradation: Economical substrates, such as industrial effluents from various sectors, would likely provide the most sustainable alternatives, but the quality of the finished product still remains as a deciding factor. The downstream processing conditions and the kind of solvent employed may have a significant impact on the thermal properties and flexibility of the obtained PHB. Utilizing different mechanical or chemical methods, combining with other polymers, adding functional groups, creating copolymers and adding additives are just a few ways to change the properties of PHB (nucleating agents, plasticizers, photostabilizers). The manufacturing of blends through combining and processing may be a practically viable and quicker approach to obtaining interesting properties of material related to copolymer manufacturing. The low crystallinity and amorphous characteristics of PHB speed up the degradation process of the polymer. Due to the polymer chain's 3HHx moieties deteriorating more quickly than 3HB moieties as time has passed since soil burial [93]. In activated sludge soil, 99.08% of PHB was broken down after 20 days, however, only 7.09% of PHB was broken down in forest soil, according to Altaee *et al.* [94] with further advancements, a variety of PHB-action technologies are potentially available that satisfy the bulk of requirements while suppressing crude analogs.

The polymer chain becomes more hydrophobic when the expansion phase kicks off, which results in the formation of a granular structure inside the cell. Enzyme PHB synthase, which is hydrophilic, binds to the chain of the ends to create a sheath that surrounds the granule and separates the polymer from the liquid cytosol. The substrate is then withdrawn from the cytosol and synthase adds to the chain to continue polymerization. Chain lengths could increase to almost 10,000 monomers. As the chains become longer, the granules are squeezed closer together until they eventually unite to occupy the cell. Production is constrained by cellular volume until sufficient substrate is present, at the point at which it can be integrated into the polymer. The 7-15 granules in *Alcaligenes eutrophus* cells have a diameter of 0.21-0.54 µm. The bacterial cell's PHB granules are in a movable, amorphous condition that makes depolymerase access to them easy. Degradation, therefore, happens concurrently with polymerization. Because of the monomers' extreme stereoregularity, the crystalline changes when PHB is extracted from the cells [95]. In general, PHA breakdown does not produce any dangerous intermediate byproducts. PHB can be degraded easily, as many bacteria have intracellular PHB depolymerases to control intracellular PHB degradation and utilization, resulting in a greater number of studies investigating PHB-degrading bacteria compared with other bioplastics [96]. The end product of PHB degradation is acetyl-CoA, which enters into either PHB synthesis pathway or Krebs cycle (Fig. 3) [97].

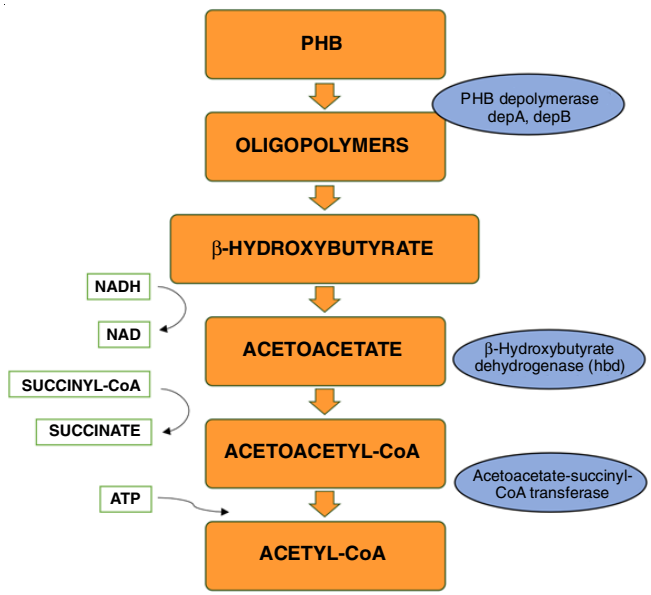

Fig. 3. Schematic representation of PHB degradation pathway

Microbial biopolymer PHB in medical application

PHB biopolymer as drug carriers: A novel nanofibrous wound dressing was prepared by Amini *et al.* [98] from polyhydroxybutyrate/chitosan (PHB/CTS) for the controlled drug delivery of the antibiotic gentamicin for the use of post-surgical ulcers which resulted in an immediate and a sustained release of about 24 h and 1 week. The potential of rifampicin-loaded PHB as chemo-embolizing drugs was also explored. It was additionally discussed how PHB, a carrier for antibiotics, is frequently administered to prevent implant-related and recurrent osteomyelitis. Nanoprecipitation, interfacial polymerization and emulsion polymerization were the main methods employed to prepare PHB nanoparticles for nanoformulation and drug administration. To ensure the delivery of pharmacologically important substances to the desired or targeted location, biocompatible systems have been developed over a few decades at an optimum and easing rate using engineered nanoparticles, microspheres and microcapsules, which are prepared from the PHA. It is common practice to deliver medications such as antibiotics, anesthetics, vaccines, anti-inflammatory agents, hormones, anticancer therapies and steroids, using a variety of microcapsules and microspheres. SCL-PHB polymers have gained a lot of attention as drug carriers because of their exceptional porosity, crystallinity and hydrophobicity, which promote

the release of the pharmaceuticals that are encapsulated without degrading the carrier polymer [83]. As a targeted drug delivery method for the treatment of breast cancer, gemcitabine-loaded polyhydroxy butyrate-coated magnetic nanoparticles (Gem-PHB-MNPs) were synthesized and characterized for the first time. The gemcitabine loaded PHB-MNPs were about two-fold more cytotoxic than free gemcitabine when compared to the SKBR-3 and MCF-7 cells, whereas the gemcitabine free PHB-MNPs were not cytotoxic. Under the influence of a magnetic field, it was shown that PHB-MNPs possess *in vitro* targeting ability [99]. There has been an investigation into the use of PHB nanoparticles to deliver, enhance the bioavailability and improve the efficacy of ursolic acid (a phytochemical compound) against cancer cells (HeLa). Using a UV-visible spectrophotometer and ursolic acid, researchers were able to determine that PHB nanoparticles had a 54% encapsulation rate *in vitro*. The cytotoxicity results indicated the maximum efficiency at the 96th hour of the PHB-loaded ursolic acid [100]. Curcumin-encapsulated PHB nanoparticles were tested for sustained curcumin administration. The nanoparticles' drug encapsulation efficiency and *in vitro* drug release were examined. The LC_{50} values of the majority of PHB-curcumin-loaded nanoparticles were found to be between 10 and 20 μg/100 μL and the anticancer activity of curcumin-loaded PHB nanoparticles was validated by AO/PI staining and the mitochondrial depolarization experiment. Particles were discovered to release drug for more than 300 min in an acidic environment, inducing apoptosis in cancer cells [101].

Biomedical application of PHB in tissue engineering: *In situ* and *ex situ* synthesis of hydroxyapatite has successfully been used to functionalize poly(3-hydroxybutyrate), obtained from sugar cane and beet agro-waste, to promote osteogenesis. MC3T3-E1 cell adhesion and proliferation were supported by initial *in vitro* experiments on poly(3-hydroxybutyrate)/hydroxyapatite composite scaffolds without any toxicity. Additionally, the composites promoted osteoblastic differentiation of MC3T3-E1 cells based on their morphological assessment. This aspect is further supported by the evaluation of the early osteogenic markers. The HA-loaded samples showed the highest ALP production and morphology typical of terminal differentiation osteoblasts [102]. Cells are grown *in vitro* on biopolymers in tissue engineering to create tissue for implantation [103]. Prior to being absorbed into the human body, foreign biomaterials often require a high level of biocompatibility. Biocompatibility is greatly influenced by the shape, surface porosity, chemistry of the biomaterials and the environment of the tissue [104]. Polyhydroxyalkanoates (PHAs) have exhibited strong potential as biomaterials for medical implants. Neural stem cells (NSCs) produced on or in PHA scaffolds, for instance, may help treat damage in the central nervous system (CNS) and PHB appears to have the greatest potential to encourage NSC differentiation into neurons among the PHA family members [87]. The solvent-casting process was used to create PHB nanocomposites successfully by using different OMMT loading wt.%. In comparison to the pure PHB polymer, the resultant nanocomposites showed increased thermal and mechanical stability. On the surface of the polymer, the scattered OMMT created pores. Blended films were subjected to *Bacillus subtilis* and PBS buffer as part of a biodegradation investigation. Comparing the 5 wt.% and 7 wt.% blended films to pure PHB film revealed that the degradation percentage improved when the nanoclay percentage was raised. In contrast to all other blended polymer films, the 5% blend increased cell proliferation according to cytotoxicity experiments, which also revealed greater cell viability and a higher cell proliferation percentage (16.27%) [105]. Cellulose nanofibers (CNFs) as a nano-additive reinforcer were selected to prepare a polyhydroxybutyrate (PHB) based nanocomposite mat. The PHB/CNF (PC) scaffold properties, prepared *via* the electrospinning method, were investigated and compared with pure PHB. To prepare a polyhydroxybutyrate (PHB) based nanocomposite, cellulose nanofibers (CNFs) were used as a nano-additive reinforcer. The properties of PHB/CNF (PC) scaffolds produced by electrospinning showed in crystallinity from 46 to 53%, the obtained results demonstrated improved water contact angle from 120º to 96º, appropriate degradation rate upto 25% weight loss in two months, prominent biomineralization (Ca/P ratio around 1.50) and an 89% increase in PHB-cellulose nanofibers based nanocomposite toughness factor when compared with pure PHB. Not only the MTT experiment showed improved human osteoblast MG63 cell viability on nanocomposite, but DAPI staining and SEM data verified the more probable cell spreading in the presence of cellulose nano-additive. These enhancements demonstrate that the PHB-based cellulose nanofiber nanocomposite composition has essential performance in the field of bone tissue engineering [106]. Electrospun PHB-starch/HNTs (halloysite nanotubes) fibrous scaffold for long-term applications such as cartilage regeneration. Tensile strength increased to 4.21 0.31 MPa when HNTs were added to enhance chondrocyte cell development. HNT incorporation resulted in surface hydrophilicity and *in vitro* breakdown. The MTT assay and cell attachment of chondrocyte cells on 2 wt.% HNTs incorporated into PHB-starch fibres shown that HNTs incorporation can support cell growth and adhesion without toxicity for biomedical applications [107]. According to the study, one-dimensional nanostructures (such as nanotubes, nanowhiskers and nanowires) are the most effective in enhancing mechanical properties due to their complexation and interaction with the host polymer. Human tissue scaffolds are made of biomaterials like agarose, chitosan, collagen, gelatin, hyaluronic acid, alginate, cellulose and fibrin, which are used to treat spinal cord injury [108]. Recent studies on the effectiveness of PHBHHx nanofiber matrices in repairing neurons suggested that these nanofibers could be used to treat neural stem cell (NSC) synaptogenesis and damage to the central nervous system (CNS) [109].

PHB as biosensor in cancer diagnosis: With the addition of acetic acid/chloroform/formaldehyde, the normal mammary epithelial cells (PCS-600-010) and metastatic breast cancer cells (T47D) were bound above the PHB sheet. According to the contact angle image, normal mammary epithelial cells did not show strong adhesion to the PHB sheets, however the breast cancer cells exhibited. The microscopic imaging is an excellent technique for visualizing malignancies. Because normal cells are neutral and cancer cells emit positive/negative charge,

thereby no attraction was visualized between normal cells and the PHB sheet [54]. As a result, the research sheds new insight into the use of PHB sheets for the detection of cancer cells. Given that the particular cancer protein interacts favourably with the PHB molecule and exhibits surface adhesion qualities, this study was perfectly correlated with the *in silico* findings [110]. PHA and its copolymers are favoured as a particular bio-material for biomedical applications due to their low cost and high conductivity to electricity [111].

The PHA graft graphene displays an excellent electrical conductivity and a greater degradation temperature than PHA in its pure state [112]. Nanomaterials derived from PHA grafts are utilized in implantable technology and conduits for nerve repair. The PHB inclusions containing a ferritin-derived iron-binding peptide and a protein A-derived antibody-binding Z domain were self-assembled using genetically modified *E. coli*. The nanobeads can precisely bind biomarkers in complex mixtures, allowing for rapid magnetic separation and increased electrochemical detection of cancer biomarkers such as cancer cell exosomes and methylated DNA [113]. PHB was supplemented for five weeks old rat model and tested for its activity against colorectal cancer. According to studies, it may reduce down on tumour growth by 58.1% and polyp formation by 48.1%. It is demonstrated as a valuable nutraceutical compound and acts as a prebiotic by increasing the number of beneficial microbes in the gut flora [114]. The PHB-HV microspheres loaded with $Ho(acac)_3$ (holmium acetylacetonate) accumulated Ho(III) on their surfaces but remained stable over time, as no expressive release of Ho(III) was found after a 9 day exposure to sodium phosphate buffer, which makes it suitable contrast agent for the magnetic resonance (MR) images by emitting beta-particles in tumor tissues [115]. The electrostatic interaction of negatively charged survivin antisense oligonucleotide (Sur-ASON) and positively charged PHB-*b*-PDMAEMA (PHB-P) co-polymer, followed by the induction of thermosensitive PF127 hydrogel, resulted in the development of a gene delivery platform. The Sur-ASON/PHB-P/PF127 hydrogel was found to be highly successful in improving the therapeutic effects of Sur-ASON while limiting degradation and the possi-bility of adverse effects *in vivo* and this unique hydrogel could enable regulated gene release for up to 16 days [116].

Biopolymer PHB as antifouling agents: Historically, toxicants like copper and tributyltin have been added to paint matrices by antifouling chemical agents to avoid settling by gently leaching the biocide from the surface layer. Other non-toxic techniques include adding naturally occurring antifouling substances originating from bacteria, algae, sponges and actinomycetes to coatings; these substances are not yet commercially available, though. To describe the effects of marine biofouling systems, PHB nanocomposite made from marine microorganisms can be produced using metal. Therefore, PHB is a promising material for producing non-toxic, eco-friendly antifoulants [117]. To increase its anti-adhesion capabilities, a hydrophobic polydimethylsiloxane matrix was substituted with biopolymer, poly(3-hydroxybutyrate-*co*-3-hydroxy valerate) and an amphiphilic system was created by adding PEG or PHBHHx-*b*-PEG copolymer. The physicochemical features of PHBHV-based coatings, as well as static adhesion tests on a marine *Bacillus* sp. and *Phaeodactylum tricornutum*, are compared to those of PDMS and PEG-modified PDMS coatings. The PHBHV/PHBHHx-*b*-PEG combination demonstrated antiadhesion action, indicating a promising alternative as an antifouling agent [118]. Combining the antibacterial capabilities of antibiotic-loaded poly(3-hydroxybutyrate) (PHB) microspheres, as well as poly-(ethylene glycol) (PEG), which acts as an antifouling agent, along with titanium as the implant base material was used to develop biomaterials with antibacterial activity [119].

Conclusion and outlook

In this review, the utilization of microorganisms has revealed their capacity for polyhydroxybutyrate (PHB) synthesis. By replacing synthetic polymers that are not biodegradable, polyhydroxybutyrate significantly contributes to the development of a sustainable environment. The cost-effective production of biodegradable polymers is especially important for breaking into the thermoplastic dominated industry. A drop in production costs will broaden the spectrum of applications for those biodegradable polymers consequently increasing their competitive value. The primary properties of such biopolymers, such as their biodegradability and biocompatibility, have facilitated their wide use in the biomedical industry, aquaculture and antifouling. There are few methods available in terms of commercially viable generation of PHB from actinobacteria and halophiles. The present review paper has successfully generated interest among academics and microbiologists about the PHB biopolymer, potentially paving the way for innovative applications across several fields. The leading candidates for the upcoming generation of multi-fit biopolymers consist of environmentally sustainable biomaterials.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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