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Production System for Biodegradable Polyester Polyhydroxybutyrate by *Corynebacterium glutamicum*

NOTES

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A biosynthetic pathway for poly(3-hydroxybutyrate) [P(3HB)] production by *Corynebacterium glutamicum* was developed by introducing the *phbCAB* operon derived from *Ralstonia eutropha*. P(3HB) synthase activity was detected in this recombinant *C. glutamicum* carrying a cell surface protein gene promoter. Intracellular P(3HB) was microscopically observed as inclusion granules and its content was calculated to be 22.5% (w/w) with a number average molecular weight of 2.1×10^5 and a polydispersity of 1.63.

Poly-3-hydroxybutyrate [P(3HB)] is a biologically produced polyester that has received much attention as a biodegradable polymer and can be produced from biorenewable resources. P(3HB) and its derivatives have many attractive properties. They can be used as bulk commodity plastics, in fishing lines, and medically, because of their biodegradability and biocompatibility. Despite the apparent benefits of using P(3HB) as a replacement for petrochemical-derived plastics, the use and distribution of P(3HB) have been limited by production cost. This problem is currently being addressed by engineering enzymes involved in P(3HB) production (1, 2).

Although several genetic engineering approaches to improve P(3HB) productivity have been reported, they have been used in only gram-negative bacteria including *Escherichia coli*, *Ralstonia eutropha*, and *Pseudomonas* strains (3). For the medical or food contact applications of P(3HB), bacterial host selection for P(3HB) production should be carefully considered, because gram-negative bacteria produce poisonous substances, i.e, endotoxins. The level of contaminated endotoxins from gram-negative bacteria can be reduced to some extent during P(3HB) preparation by chloroform or NaOH treatment, but a trace amount of endotoxins still remain (4). To avoid such harmful contaminants, the use of gram-positive bacteria for P(3HB) production is preferable. Gram-positive bacteria, particularly native P(3HB)-producing *Bacillus* strains, have long been studied for efficient P(3HB) production but their productivities are extremely low (5). However, P(3HB) production by recombinant gram-positive bacteria has rarely been reported.

Corynebacterium glutamicum is an aerobic, gram-positive, nonsporulating bacterium that has been extensively employed for the industrial production of several amino acids, based on classical metabolic engineering, that have been used in food,

feed and pharmaceutical products for several decades (6, 7). The metabolic pathways and whole genome sequence of this bacterium have recently been reported (<http://www.genome.ad.jp/kegg/pathway.html>; 8). Hence, *C. glutamicum* is an attractive candidate host for P(3HB) production at the genome-wide level. Acetyl-CoA generated from sugars via glycolysis is a key compound for the biosynthesis of various biomaterials such as amino acids and fatty acids. Acetyl-CoA is also a starting substance required for the P(3HB) biosynthetic pathway consisting of 3-hydroxybutyryl-CoA (3HB-CoA) synthesis [catalyzed by β -ketothiolase (PhbA) and NADPH-dependent acetoacetyl-CoA reductase (PhbB)] and the polymerization of 3HB-CoA to P(3HB) [catalyzed by P(3HB) synthase (PhbC)]. Figure 1A shows the metabolic linkage between the glutamate synthetic pathway and our proposed P(3HB) synthetic pathway. As a metabolic design, we tried to channel acetyl-CoA into an artificial P(3HB) synthesis pathway. To this end, an artificial P(3HB) synthetic pathway was developed in *C. glutamicum* by introducing an *R. eutropha*-derived *phbCAB* operon, as shown in Fig. 1B.

E. coli JM109 was used for genetic manipulation and grown at 37°C in Luria-Bertani (LB) medium. When necessary, ampicillin (100 μ g/ml) or kanamycin (50 μ g/ml) was added to the medium. *C. glutamicum* ATCC13869, provided by Dr. K. Yokoyama of Ajinomoto Co. Inc., was used for transformation and P(3HB) biosynthetic gene expression. *C. glutamicum* was transformed by electroporation as described previously (9). Three media were evaluated for the efficient production of P(3HB) in *C. glutamicum*: LB medium, a nutrient-rich (CM2G, 10) medium and a minimal (MMTG, 10) medium often used for accelerating protein production in *C. glutamicum*. P(3HB) production was observed only for MMTG medium. Thus,

MMTG medium was used in the following experiments. Kanamycin was added to the medium at 50 $\mu\text{g/ml}$. The *C. glutamicum* transformant was cultivated at 30°C for 72 h. Cells harvested were by centrifugation at 14,000xg for 2 min and disrupted by sonication (4 sec, 15 times) on ice. Cell debris was precipitated by centrifugation at 14,000xg for 15 min to prepare whole cell extract. The P(3HB) synthase (PhbC) activity of the whole cell extract was determined at 25°C by measuring the amount of released CoA during the polymerization of 3HB-CoA in absorbance at 412 nm, as described previously (11).

The P(3HB) content of the transformant was quantitated by high-performance liquid chromatography at 60°C using an Aminex HPX-87H ion exclusion column (7.8 mm I.D. x 300 mm; Bio-Rad Laboratories, Hercules, CA, USA). P(3HB) was converted to crotonic acid with concentrated sulfuric acid and diluted 10-fold with 0.014 N H₂SO₄. Samples were filtered through a 0.45- μm PTFE membrane (Advantec, Tokyo) and eluted with 0.014 N H₂SO₄ at a flow rate of 0.7 ml/min. The absorbance of crotonic acid was measured at 210 nm (12). The molecular weight of P(3HB) was determined by gel permeation chromatography (GPC) at 40°C using a Jasco GPC-900 system equipped with tandem columns of TSK gel GMH_{HR}-M (7.8 mm I.D. x 300 mm; Tosho, Tokyo) and Shodex XF-804L (8 mm I.D. x 300 mm; Showa Denko K. K., Tokyo). Chloroform was used as an eluent at a flow rate of 0.8 ml/min, and calibration was performed using standard polystyrene samples. For transmission electron microscopy (TEM), *C. glutamicum* cells were fixed in 2% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 1 h and postfixed in 2% osmium tetroxide for 30 min. The samples were dehydrated in a graded ethanol series and embedded in Epon812 epoxy resin. Ultrathin sections were prepared, poststained with uranyl acetate

and lead acetate, and examined using an electron microscope (JEM-2010; Jeol, Tokyo).

To express the P(3HB) biosynthesis genes from *R. eutropha* in *C. glutamicum*, two expression plasmids, pGEM-*phbCAB* and pPS-*phbCAB*, were constructed, as shown in Fig. 1B. These two plasmids can be used in *C. glutamicum* and *E. coli* as shuttle vectors. An 5.0-kb *SmaI-BamHI* fragment containing the *phb* promoter, P(3HB) biosynthesis (*phbCAB*) genes, and the *phb* terminator of *R. eutropha* from pGEM-*phbCAB* (13) was inserted into the *KpnI-BamHI* site of the pPSPTG1 vector (10) after being blunt-ended at the *KpnI* site. The resulting expression plasmid pGEM-*phbCAB* was constructed so that the *phbCAB* genes could be expressed under the control of the *phb* promoter. An expression plasmid, pPS-*phbCAB*, was constructed using a 4.3-kb blunt-ended *Csp45I-BamHI* fragment of pGEM-*phbCAB* containing *phbCAB* and the *phb* terminator subcloned into the blunt-ended *BstEII-BamHI* site of pPSPTG1. The expression of the *phbCAB* operon was controlled by the promoter for the gene (*cspB*) encoding a cell surface protein B; the *cspB* promoter has been used for the high production of the protein of interest in *C. glutamicum* (10). Both *C. glutamicum* transformants harboring pGEM-*phbCAB* and pPS-*phbCAB* grew similarly in MMTG medium, but PhbC activity in the whole cell extracts was detected only in the pPS-*phbCAB* transformant (data not shown). These results indicate that the *R. eutropha*-derived *phbCAB*-specific promoter is not functional in *C. glutamicum* cells, whereas the *cspB* promoter can be operated in *C. glutamicum* for the expression of the *phbCAB* operon from *R. eutropha*. Monomer supplying enzyme genes, *phbA* and *phbB*, are expected to be expressed in *C. glutamicum* by the formation of an operon with *phbC*.

The culture conditions of the *C. glutamicum* transformant with pPS-*phbCAB*

for P(3HB) production were investigated in terms of the initial pH of MMTG medium and cultivation temperature. Maximum cell growth after 72-h cultivation was observed at 27°C and pH 7.5, whereas maximum P(3HB) production was obtained at 30°C and pH 7.5. Therefore, cultivation for P(3HB) production of was performed at 30°C and pH 7.5. The time courses of cell growth and P(3HB) production were plotted to overlap with each other, as shown in Fig. 2. Essentially, a synchronized pattern was obtained between cell growth and P(3HB) production during the course of cultivation. Cell growth reached 12.5 mg/ml at 36 h, whereas P(3HB) content linearly increased up to 48 h and plateaued at about 22.5% (w/w), which continued until 96 h of cultivation. Gas chromatographic and nuclear magnetic resonance spectroscopic analyses based on previous procedures (14) revealed that the accumulated polymer in the recombinant *C. glutamicum* is a homopolymer consisting of only 3HB-monomer units (data not shown).

Intracellular P(3HB) was observed as inclusion granules within *C. glutamicum* cells harboring pPS-*phbCAB* by TEM, as shown in Fig. 3. The shape and size of the fully grown cells which accumulated P(3HB) granules were unchanged, contrary to the filamentous morphogenesis often observed in recombinant *E. coli* producing P(3HB) (15).

By GPC, the synthesized P(3HB) prepared from 72-h-cultivation cells showed a number average molecular weight (M_n) and a polydispersity of 2.1×10^5 and 1.63, respectively, which differed from those of P(3HB) (M_n of 1.8×10^6 and polydispersity of 1.8) synthesized in the recombinant *E. coli* harboring the *phbCAB* operon from *R. eutropha*, as shown in Table 1. The differences in these properties between P(3HB)s synthesized by both bacterial strains could be due to the differences

between individual physiological features such as monomer supplying ability and the expression levels of P(3HB) biosynthetic genes. Although the P(3HB) content of the recombinant *C. glutamicum* was lower than that of the recombinant *E. coli*, the cell density of the recombinant *C. glutamicum* was almost 4-fold that of the recombinant *E. coli* (Table 1). These results indicate that P(3HB) production by recombinant *C. glutamicum* is more efficient than that by recombinant *E. coli*. The P(3HB) production system in *C. glutamicum* can be improved by analyzing the metabolic flux of acetyl-CoA or the polarity effect of the promoter used.

This is the first report on an endotoxin-free production system of P(3HB) in recombinant *C. glutamicum* by constructing an artificial P(3HB) biosynthetic pathway channeling acetyl-CoA. This beneficial system will facilitate further next-generation research studies such as those on the biosynthesis of 3HB-based copolymers with desirable properties and the industrial production of biopolymers together with amino acids from renewable carbon sources.

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Figure legends

FIG. 1. (A) Metabolic linkage between glutamate synthetic pathway and P(3HB) synthetic pathway, and (B) structures of constructed plasmids pPGEM-*phbCAB* and pPS-*phbCAB*. Each plasmid carries a single operon (*phbCAB*) consisting of three genes, *phbC* (P(3HB) synthase), *phbA* (β -ketothiolase), and *phbB* (NADPH-dependent acetoacetyl-CoA reductase) with the *phb* terminator. The expression of the operon was regulated by the *phb* promoter (*Pphb*) for pPGEM-*phbCAB* and the *cspB* promoter (*Pcsp*) for pPS-*phbCAB*. Km^r, Kanamycin resistance gene; ori, replication origin; P, promoter; C, *phbC*; A, *phbA*; B, *phbB*; T, terminator.

FIG. 2. Time courses of cell growth (dry cell weight, DCW; closed circles) and P(3HB) content (open circles) during cultivation of recombinant *C. glutamicum* cells harboring pPS-*phbCAB*.

FIG. 3. TEM images of recombinant *C. glutamicum* cells harboring pPGEM-*phbCAB* (A) and pPS-*phbCAB* (B). P(3HB) accumulation was observed as granules in the cytoplasm of the cell (B). Bars indicate 0.2 μ m (A) and 0.5 μ m (B), respectively.

Table 1 P(3HB) biosynthesis of by *C. glutamicum* and *E. coli* recombinants

Plasmid (strain)	M_w	M_n	Polydispersity (M_w/M_n)	Dry cell weight (g/l)	P(3HB) content (%,w/w)	Ref.
pPS- <i>phbCAB</i> (<i>C. glutamicum</i>)	3.4×10^5	2.1×10^5	1.63	12.5	22.5	This study
pGEM- <i>phbCAB</i> (<i>E. coli</i>)	3.2×10^6	1.8×10^6	1.8	3.2	33	16

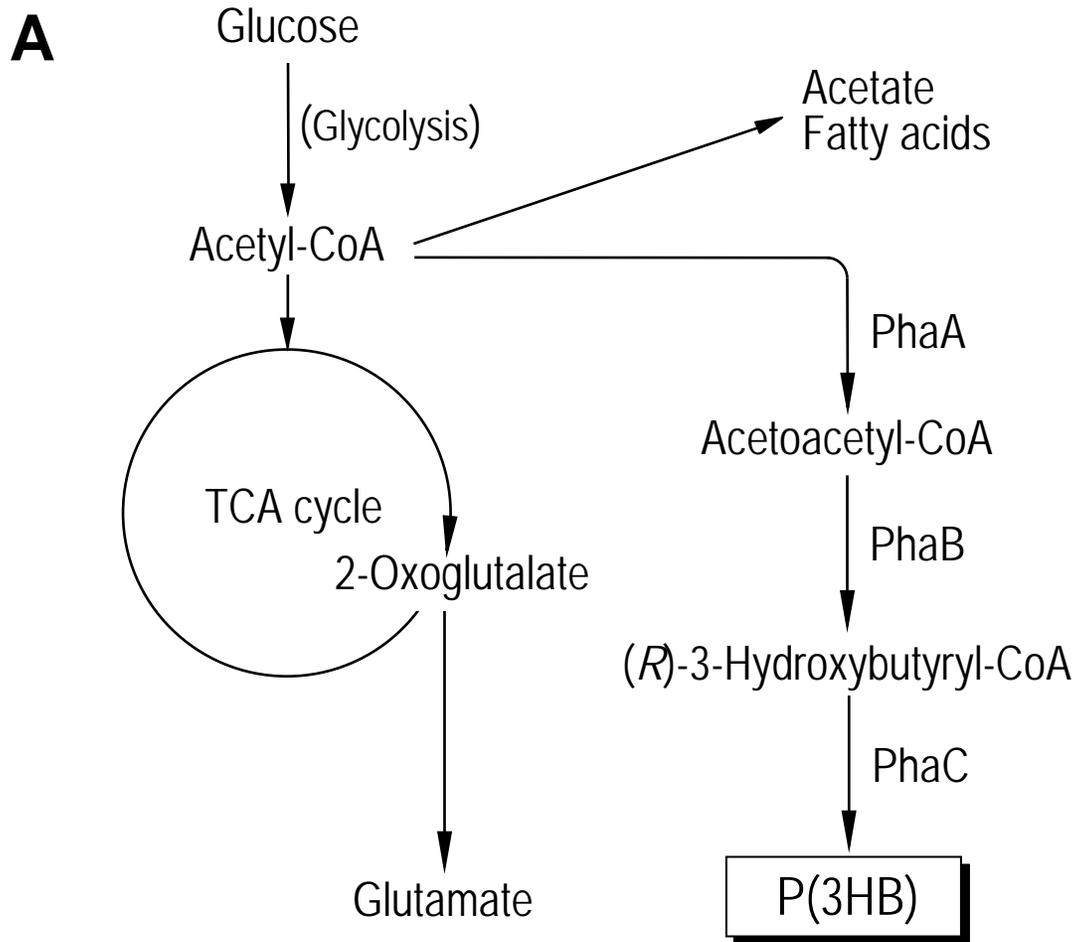


Fig. 1 (A). Jo SJ. *et. al.*

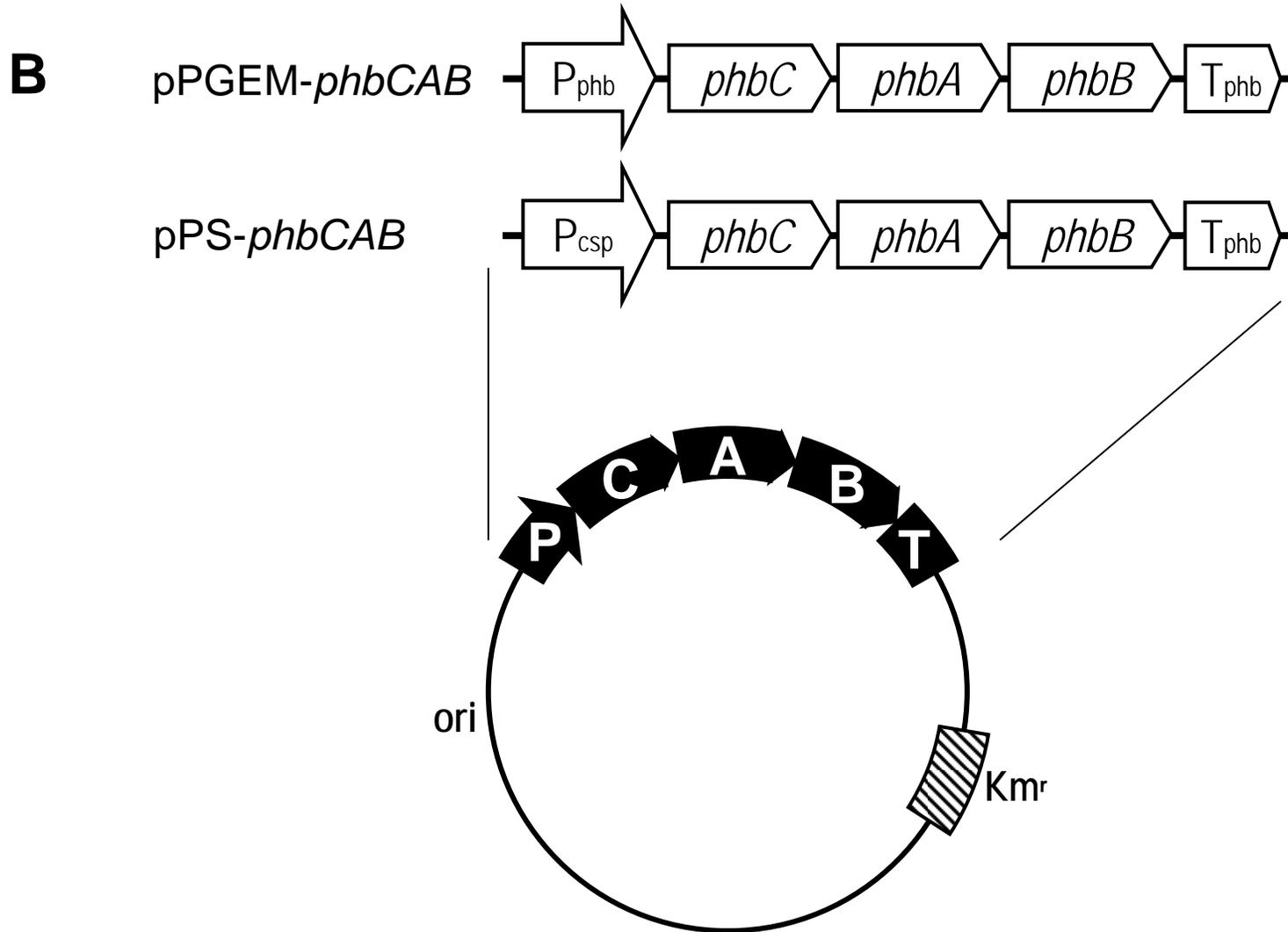


Fig. 1 (B). Jo SJ. *et. al.*

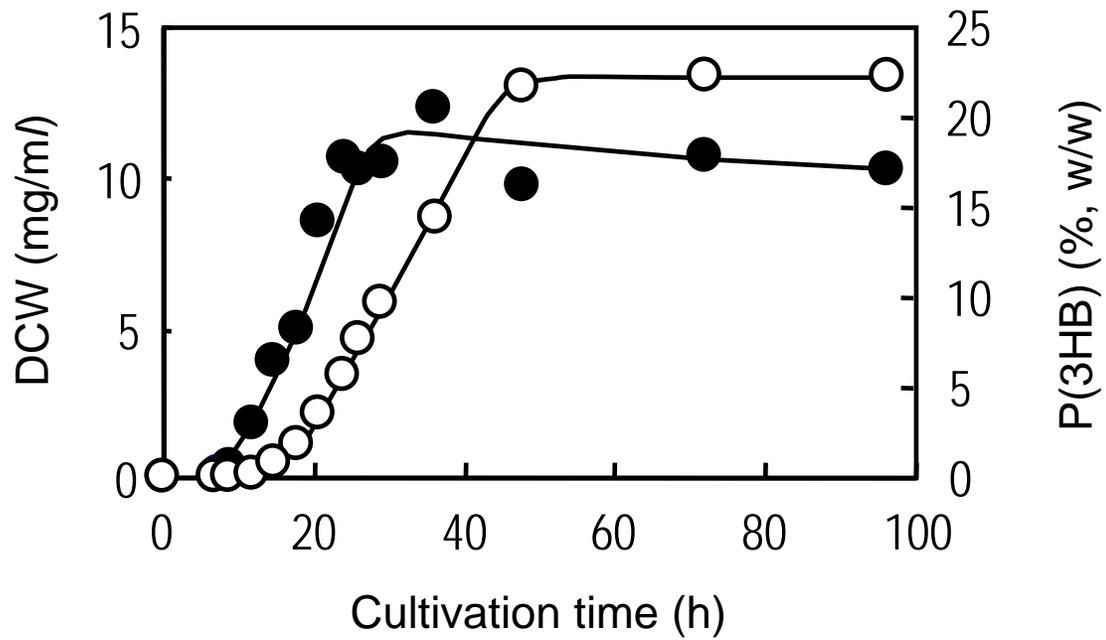


FIG. 2. Jo SJ. *et. al.*

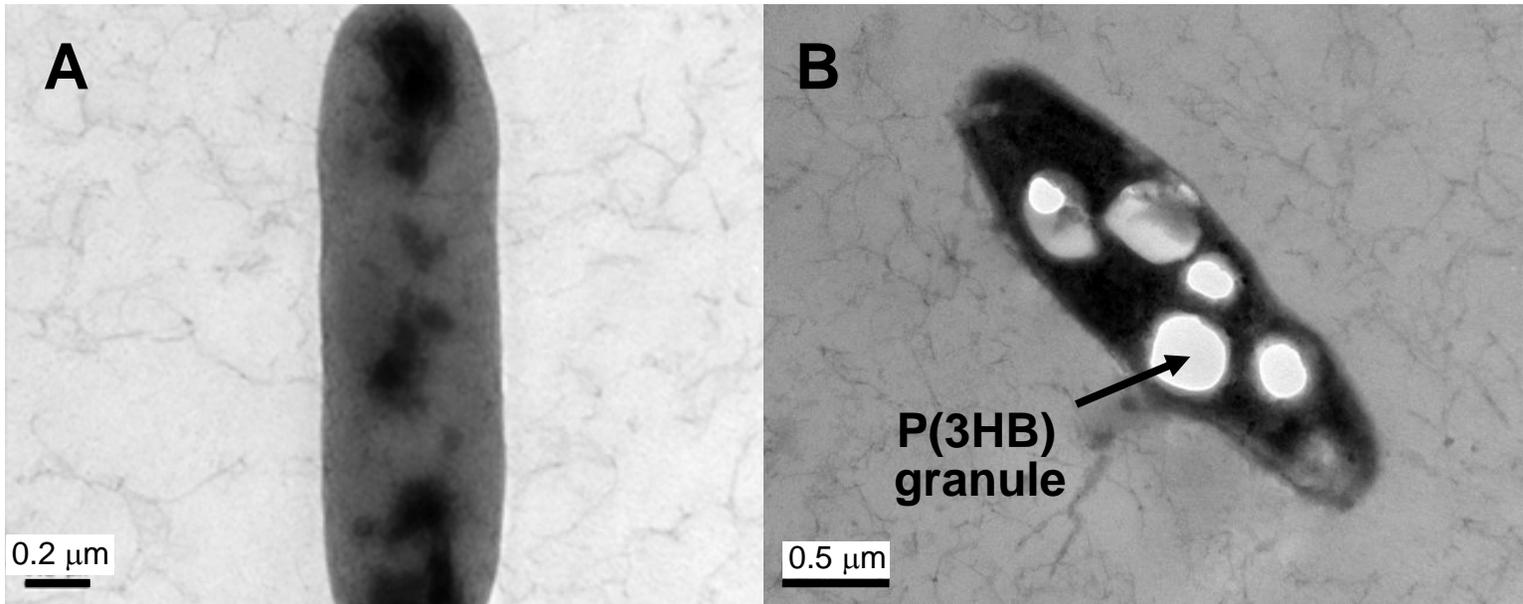


FIG. 3. Jo SJ. *et. al.*