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Development of Cultivation Media for Polyhydroxyalkanoates Accumulation in Bacterial Cells Isolated from Cassava Pulp

[1]Onuma Chansatein, [3]Hathairat Urairong and [2]Sureelak Rodtong
[1]School of Biology, [2]School of Microbiology, Institute of Science,
Suranaree University of Technology, 30000 Nakhon Ratchasima, Thailand
[3]Biotechnology Research and Development Office, Department of Agriculture,
Ministry of Agriculture and Cooperatives, 12110 Pathum Thani, Thailand

Abstract: Polyhydroxyalkanoates (PHAs) are biopolymers efficiently used as biodegradable plastics to replace environmentally unfriendly petroleum-derived plastics. The polymers can be synthesized by a wide range of microorganisms. Bacteria accumulate PHAs under conditions of nutrient stress particularly nitrogen or phosphorus limitations. In this study, cultivation media were developed for detecting a number of bacteria isolated from cassava pulp for their PHA production capability by modifying media described by potential references. Both complex and minimal media were developed when cultured at 35°C for 48 h. The isolates were tested for the presence of PHA accumulation by straining with 1% Nile blue A and observed under the fluorescence microscope at excitation wave length of 650 nm. Transmission Electron Microscope (TEM) was used for comfirmation of PHA granules accumulation in bacterial cells. *Alcaligenes eutrophus* (TISTR 1095) and *E. coli* (TISTR 527) were used as the positive and negative control of PHAs-producing strain, respectively. This is the first report for the suitable media for detecting of PHAs-producing bacteria isolated from cassava pulp.

Key words: Development, cultivation media, polyhydroxyalkanoates, accumulation, *E. coli*, Thailand

INTRODUCTION

Various microorganisms can produce the biopolymers, Polyhydroxyalkanoates (PHAs), intracellular granular forms undernutrient-limited. The polymers serve as reserves of carbon and reducing equivalents to preserve cell survival during stress conditions (Sudesh *et al*., 2000). PHA granules in bacterial cells could be detected using specific cultural media and staining with lipophilic dyes such as Nile blue, Nile red or Sudanblack B and TEM was used for comfirmation of PHA granules accumulation in bacterial cells (Sudesh *et al*., 2000; Zinn *et al*., 2001). PHAs-producing bacteria have been reported to be found in various environments such as wastewater, industrial waste, municipal waste, soil, compost, hot spring water, fresh water and marine water (Sudesh *et al*., 2000). However, PHAs-producing bacteria isolated from cassava pulp had not yet been studied. Cassava pulp is the solid waste produced as a consequence of starch production. This pulp contains a high starch content (50-60% dry basis) which yields around 1.5-1.8 million metric ton annually in Thailand. The main application for the large quantities of waste material produced each year, after drying is as animal feed or fertilizer (Sriroth *et al*., 2000). The strains could produce PHAs from biowaste (cassava pulp) as cheap source of substrate.

MATERIALS AND METHODS

Samples collection: Sources of bacteria isolated from cassava pulp samples were the Microbial Culture Collection and Applications Research Unit, Institute of Science, Suranaree University of Technology and new isolates from modified cassava starch industrial factories and sun drying field for cassava pulp in Northeastern Thailand.

Bacterial isolation: About 25 g of cassava pulp samples were mixed with 225 mL of phosphate buffer pH 7.2 to make the 1:10 dilution. The homogeneous serial dilutions of 1:10^2-1:10^8 were prepared and each of dilution was spreaded on five difference agars including Carboxymethylcellulose (CMC) agar, Thermocarboxymethylcellulose (TCMC) agar, Plate Count Agar (PCA), Trypticase Soy Agar (TSA) and Starch Agar (SA) and

Corresponding Author: Onuma Chansatein, School of Biology, Institute of Science, Suranaree University of Technology, 30000 Nakhon Ratchasima, Thailand

incubating at 35°C for 48 h. Difference size, color, edge, surface, whole colony, elevation and diameter of colony were selected for PHA detection in their cells. The selected isolates were re-streaked on the original agar medium for bacterial purification. Purified isolates were kept in 5% skim milk (final concentration) at -80°C as stock culture and using for the detection of PHAs producing bacteria.

Development of cultivation media for PHA accumulation in bacterial cells: Bacterial isolates from stock culture were re-streaked on TSA for pure culture preparation. The isolates characterized by morphological characteristics according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Then, the isolates were arranged by groups. Appropriate media for the detection of PHAs-producing bacteria were modified for each group based on methods which were described by Lee *et al.* (1994), He *et al.* (1998), Kim *et al.* (2000), Yu *et al.* (2002), Kojima *et al.* (2004), Takagi *et al.* (2004), Zheng *et al.* (2005), Full *et al.* (2006), Berlanga *et al.* (2006), Ciesielski *et al.* (2006) and Halet *et al.* (2007). Selections media, twenty bacterial isolates from cassava pulp were tested for their growth in the investigated media.

The bacterial isolates were grown in complex medium according to references (Lee *et al.*, 1994; Yu *et al.*, 2002; Tajima *et al.*, 2003; Kung *et al.*, 2007) and modified medium for this study.

Fluorescent microscopy for the detection of PHA accumulation in bacterial cells: The isolates were detected for the presence of PHA accumulation by the methods according to Ostle and Holt (1982), Lee *et al.* (1994) and Rodtong *et al.* (2008). Pure cultures of bacteria were culturing on modified complex medium incubating at 30°C for 48 h then transferred to minimal medium and incubating at the same condition. Heat-fixed smears of bacterial cells were stained with 1% Nile blue A solution and observed under fluorescence microscope (Fluorescence microscope; Olympus Model BX51TRF, Olympus Optical Co., Ltd., Japan) at excitation wave length of 650 nm. *Alcaligenes eutrophus* (TISTR 1095) was used as the positive control of PHAs-producing strains.

Percentage of PHA accumulation in bacterial cells was measured by Image-Pro Plus Version 6.0 Program (Media Cybernetics, Bethesda, MD, USA) from density of bright orange color when observed under fluorescent detector from fluorescent microscope. Bacterial isolates accumulating high content of PHAs were selected for electron microscopy detection.

Electron microscopy for the detection of PHA accumulation in bacterial cells: Transmission Electron Microscopy (TEM) was used for detecting PHAs in bacterial cells. Fixative solution was used composing of 5% glutaraldehyde, 1% Osmiumtetoxide, 0.2 M phosphate buffer in the ratio of 1:1:1. After 2 h of fixation, the bacterial cells were spun down at 8,000 rpm for 10 min using a micro centrifuge. The supernatant was removed and an additional 0.1 M of phosphate buffer 1 mL was added to wash the cell pellet for 10 min and 3 times. Uranyl acetate (4% concentration) was used for enbloc staining and incubating at room temperature in the dark for 1 h.

Uranyl acetate was then removed. The cell pellet was washed twice with 1 mL of sterile distilled water for 10 min for each wash. Acetone series (v/v) for dehydration were 20, 40, 60, 80 and 100%. The pellet was placed the 1st time in 20% acetone for 10 min and subsequently in 40, 60 and 80% acetone for 10 min and twice in 100% acetone for 10 min.

The cells were spun down after each acetone treatment to remove the supernatant. The samples were infiltrated with 100% acetone and Epon ratios of acetone and Epon in infiltration were 2:1, 1:1 and 1:2. The ratios 2:1 and 1:1 were incubated at room temperature for 1 h and then 1:2 was added and incubated for overnight at the same temperature. Each step of infiltration was removed the supernatant before changing to the next ratio. Then, low viscosity solutions were penetrated with pure Epon for 3 h.

The solutions were transferred to beam capsules containing 100% low-viscosity embedding resin. The beam capsules were placed at 60°C for 24 h to allow to embedding.

Ultrathin sections of the bacterial cells were prepared using ultra cut microtome (Ultracut RMC Boeckeler ®, Boeckeler Instruments Inc., USA) with a Diatome diamond knife.

The sections were picked up with 200 mesh copper grids coated with Formvar (0.3% [w/v] dissolved in ethylene dichloride) and a layer of carbon. Grids with specimens were put on 5% uranyl acetate droplet on a piece of parafilm extended inside closed glass petri dish in the dark for 15 min. Then washed in sterile distilled water by dipping for several times and put on tiny drop of 0.4% lead citrate which surrounding with sodium hydroxide (NaOH) pellets on a piece of parafilm extended inside closed glass petri dish for 15 min. Grids were washed again in new clean water and placed in the grid box when completely dry. The sections were examined using TEM (JEOL JEM-1230, JEOL, Japan) and recorded of TEM images.

RESULTS AND DISCUSSION

Bacterial isolates: Bacterial isolates isolated from cassava pulp, the majority of colonies were circular, entire edge, smooth surface, umbronate elevation and 0.1-0.3 cm in diameter. For cell morphology were gram-positive and negative bacteria. Sizes of these bacterial cells ranged from 0.18-0.28×0.18-0.28 to 0.77-1.05×3.67-6.38 μm. These bacteria could be grouped into 4 groups: regular, non-sporing gram-positive rod bacteria; gram-negative rod bacteria; endospore-forming gram-positive rod bacteria and gram-positive coccus bacteria.

Development of cultivation media for PHA accumulation in bacterial cells: Development of culture media for PHAs-producing bacteria was studied to obtain the appropriate media for the detection of these bacteria. Major and minor elements in complex and minimal media were investigated using data from relevant references. Twenty bacterial isolates from cassava pulp were tested for their growth in the investigated media. It was found that the suitable complex medium was composed of (per liter) 5 g yeast extract, 5 g polypeptone, 5 g tryptone, 2.5 g NaCl and 10 g glucose (Table 1). In this study, the medium could promote bacterial cell growth within 10 h of incubation. Comparison of bacterial cell growth. The suitable minimal medium was found to compose of 0.01 g $CaCl_2.2H_2O$, 0.05 g ferrous ammonium citrate, 10 g glucose, 1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 3 g Na_2HPO_4, 1 g $(NH_4)2SO_4$, 1 mL of trace element solution (0.2 g $CoCl_2.6H_2O$, 0.01 g $CuSO_4.5H_2O$, 5.56 g $FeSO_4.7H_2O$, 0.3 g H_3BO_3, 0.03 g $MnCl_2.4H_2O$, 0.03 g $NaMoO_4.2H_2O$, 0.02 g $NiCl_2.6H_2O$, 0.1 g $ZnSO_4.7H_2O$) (Table 2 and 3).

Fluorescent microscopy for the detection of PHA accumulation in bacterial cells: Comparison of PHA accumulation in bacterial cells between minimal medium from references and modified medium in this study were shown in Table 4 and Fig. 1.

Table 1: Complex medium for the detection of PHAs-producing bacteria isolated from cassava pulp

	Complex medium[a]					Medium modified for
Components ($g\ L^{-1}$)	A	B	C	D	E	this study
Yeast extract	2	5	5.0	10	5.0	5.0
Meat extract	3	-	-	5	-	-
Beef extract	-	-	2.5	-	-	-
Polypeptone	5	-	-	10	-	5.0
Tryptone	-	10	-	-	8.0	5.0
Trypticase	-	-	-	-	-	-
Peptone	-	-	5.0	-	-	-
Phytane	-	-	-	-	-	-
NaCl	2	5	-	-	2.5	2.5
$(NH_4)_2SO_4$	-	-	2.5	5	-	-
Glucose	-	10	-	-	-	10.0

[a]A: Tajima *et al.* (2003); B: Lee *et al.* (1994); C: Yu *et al.* (2002); D: Fukui *et al.* (1998); E: Kung *et al.* (2007)

Table 2: Optimized minimal medium for the detection of PHAs-producing bacteria isolated from cassava pulp

	Concentrations ($g\ L^{-1}$)[a]												Optimized
Components	A	B	C	D	E	F	G	H	I	J	K	L	medium
Agar	-	-	-	-	-	-	-	-	-	15	-	-	15
$CaCl_2$	-	-	-	-	-	-	-	-	-	0.01	-	-	-
$CaCl_2.2H_2O$	0.01	-	0.75	0.1	-	0.7	-	0.01	-	-	-	0.01	-
EDTA	-	-	-	-	-	0.1	-	-	-	-	-	-	-
Ferrous ammonium citrate	-	-	-	-	-	-	0.05	-	-	0.06	-	-	0.05
$FeSO_4.7H_2O$	0.02	-	-	-	-	-	-	-	-	-	-	0.06	-
Glucose	10	-	-	-	-	-	-	-	-	5	-	-	10
K_2HPO_4	-	-	-	-	-	0.92	-	-	5.8	-	-	-	-
KH_2PO_4	0.83	2.65	1	2.3	1.4	0.45	1.4	2.3	3.7	1.5	2.65	1.5	1
$MgSO_4.7H_2O$	-	0.4	-	-	0.3	-	0.3	-	-	-	-	-	-
100 mM $MgSO_4$	-	-	-	-	-	-	-	-	0.24	-	-	-	-
$MgSO_3.7H_2O$	0.2	-	0.2	-	-	-	-	0.25	-	-	-	-	-
$MgSO_4.7H_2O$	-	-	-	0.25	-	6	-	-	-	0.2	0.4	0.2	0.2
$NaHCO_3$	-	-	-	0.3	-	-	0.5	0.3	-	-	-	-	-
Na_2HPO_4	-	3.8	-	-	3	-	1.4	-	-	-	-	-	3
$Na_2HPO_4.7H_2O$	-	-	-	-	-	-	-	-	-	6.7	-	6.7	-
$Na_2HPO_4.12H_2O$	3.32	-	-	-	-	-	-	7.3	-	-	9.65	-	-
Na_2HCO_3	-	-	-	-	0.3	-	-	-	-	-	-	-	-
Sodium acetate	-	-	-	-	-	2.4	-	-	-	-	-	-	-
NH_4Cl	-	-	-	-	-	0.16	-	-	-	0.1	-	-	-
$(NH_4)_2SO_4$	2	-	0.75	-	3	-	15	0.66	-	-	0.5	0.5	1
$(NH_4)H_2PO_4$	-	-	-	-	-	-	-	-	5.94	-	-	-	-
Nile red	-	-	-	-	-	-	-	-	-	0.000	-	-	0.0005
Yeast extract	-	-	-	-	1	-	0.3	-	-	-	-	-	-
Na_2HCO_3	-	-	-	-	0.3	-	-	-	-	-	-	-	-

[a]A: Lee *et al.* (1994); B: Yu *et al.* (2002); C: Full *et al.* (2006); D: Ciesielski *et al.* (2006); E: Kojima *et al.* (2004); F: Halet *et al.* (2007); G: Takagi *et al.* (2004); H: Kim *et al.* (2000); I: Foster *et al.* (2005); J: Berlanga *et al.* (2006); K: He *et al.* (1998); L: Zheng *et al.* (2005)

Table 3: Optimized trace element solution for the detection of PHAs-producing bacteria isolated from cassava pulp

Components	Trace elements ($g\ L^{-1}$)[a]										Optimized trace element
	A	B	C	D	E	F	G	H	I	J	
$CaCl_2$	-	-	-	9	-	-	-	-	-	-	-
$CaCl_2.2H_2O$	-	-	-	-	-	-	-	1.67	-	-	-
$CaCl_2.H_2O$	-	-	-	-	-	-	-	-	10	-	-
$CaCl_2.6H_2O$	0.2	-	-	-	0.15	2	-	-	-	-	0.20
$CoCl_2.H_2O$	-	-	-	-	-	-	-	-	-	0.2	-
$CoSO_4.7H_2O$	-	-	5.62	-	-	-	5.62	2.81	-	-	-
$CuCl_2.2H_2O$	-	-	0.34	-	-	-	0.34	0.117	-	-	-
$CoSO_4.5H_2O$	0.01	0.002	-	-	0.03	0.1	-	-	0.03	0.01	0.01
$Fe(NH_4)SO_4$	-	0.2	-	-	-	-	-	-	-	-	-
$FeCl_3.6H_2O$	-	-	-	-	1.5	-	-	-	20	-	-
$FeSO_4.7H_2O$	-	-	5.56	9	-	-	5.56	2.78	-	-	5.56
H_3BO_3	0.3	-	0.6	-	0.15	3	0.6	-	-	0.3	0.30
$MnCl_2.2H_2O$	-	-	-	1.5	-	-	-	-	-	-	-
$MnCl_2.4H_2O$	0.03	0.005	3.96	0.15	0.12	0.3	3.96	1.98	0.05	0.03	0.03
$NaBO_4O_2.10H_2O$	-	0.002	-	-	-	-	-	-	-	-	-
$NaMoO_4.2H_2O$	0.03	0.002	0.06	-0.06	0.2	0.06	-	-	-	0.03	0.03
$NiCl_2.6H_2O$	0.02	-	0.04	-	-	-	0.04	-	-	0.02	0.02
$NiSO_4.7H_2O$	-	-	-	-	-	0.28	-	-	-	-	-
$ZnSO_4$	-	-	-	-	-	-	-	0.29	-	-	-
$ZnSO_4.7H_2O$	0.1	0.005	0.58	1.5	0.12	1	0.58	-	0.1	0.10	0.10

[a]A: Lee *et al.* (1994); B: Yu *et al.* (2002); C: Ciesielski *et al.* (2006); D: Kojima *et al.* (2004); E: Halet *et al.* (2007); F: Takagi *et al.* (2004); G: Kim *et al.* (2000); H: Foster *et al.* (2005); I: He *et al.* (1998); J: Zheng *et al.* (2005)

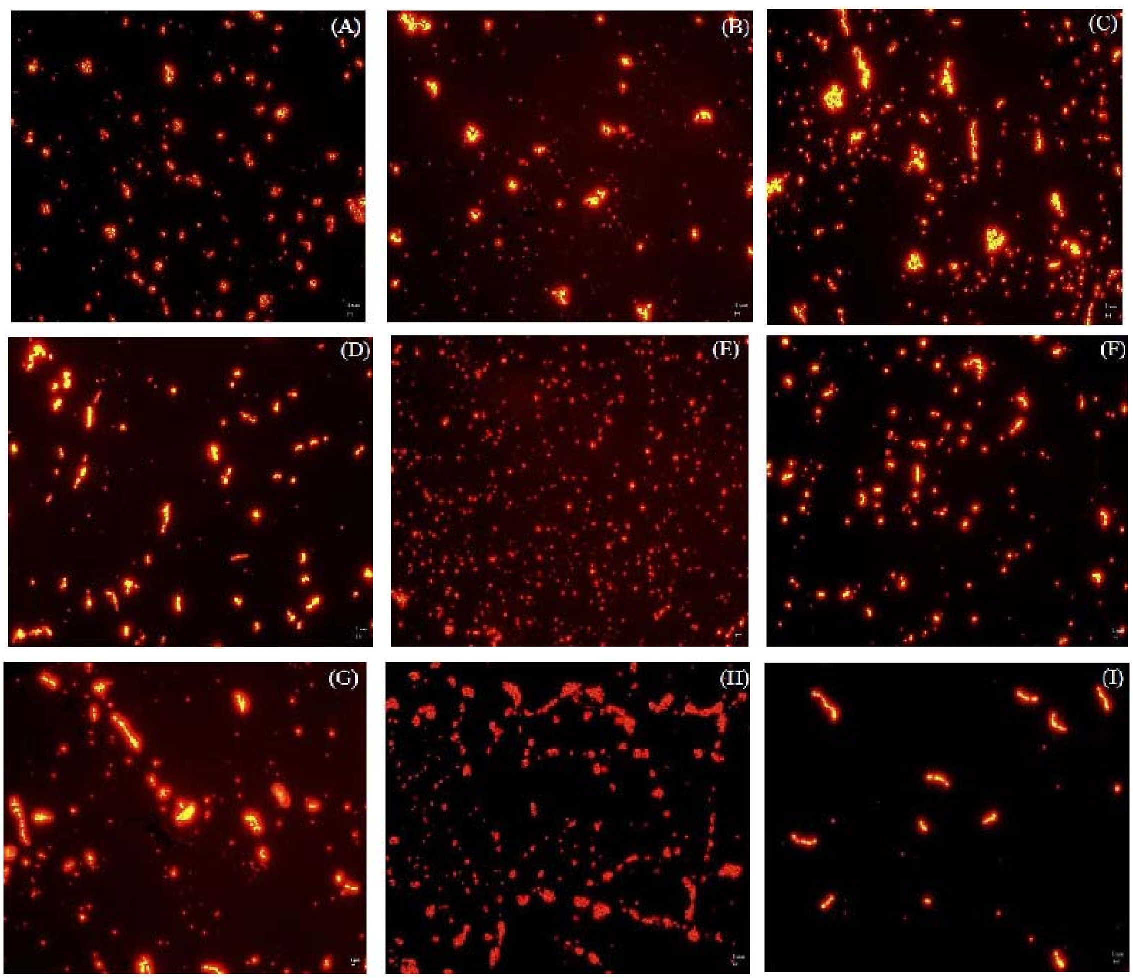

Fig. 1: Continue

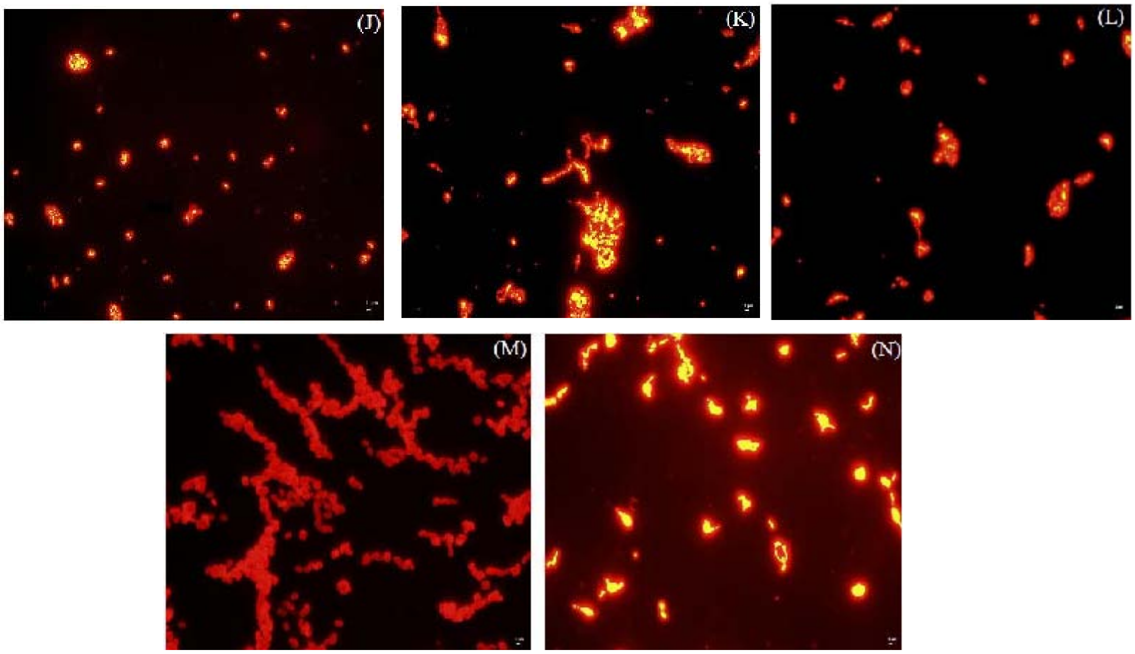

Fig. 1: PHA accumulation in bacterial cells compared between minimal medium from references: A) Lee *et al.* (1994); B) He *et al.* (1998); C) Kim *et al.* (2000); D) Yu *et al.* (2002); E) Kojima *et al.* (2004); F) Takagi *et al.* (2004); G) Foster *et al.* (2005); H) Zheng *et al.* (2005); I) Full *et al.* (2006); J) Berlanga *et al.* (2006); K) Halet *et al.* (2007); L) Ciesielski *et al.* (2006); M) Negative control (*E. coli* TISTR 527); N) Modified medium in this study

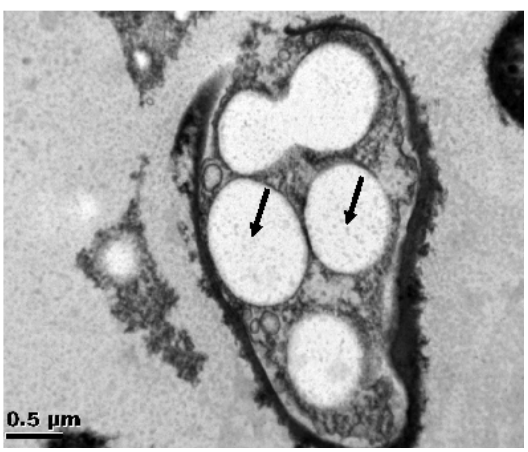

Fig. 2: TEM micrographs of granules (arrows) in cells of bacterial isolates CWC 1-5

Electron microscopy for the detection of PHA accumulation inbacterial cells: Gram-positive, rod shape and endospore-forming bacterial isolate CWC 1-5 which was accumulating high content of PHAs up to 77.6% of cell area was a representative of selected for electron microscopy detection (Fig. 2). Developed media for the detection of PHA accumulation in the bacterial cells isolated from cassava pulp were investigated for all groups, based on methods that have been previous described. Some major elements for culturing PHAs-producing bacteria isolated from cassava pulp were further optimized. Phosphate has been reported to be a crucial element stimulating PHA accumulation in bacterial cell (Shi *et al.*, 2007). Phosphate in the form of potassium phosphate (KH_2PO_4) and disodium phosphate (Na_2HPO_4) was varied in its concentrations. Phosphate that has been reported to be influence the accumulation of PHAs was

Table 4: PHA accumulation in bacterial cells compared between minimal medium from references, negative control (*E. coli* TISTR 527) and optimized medium in this study

Minimal medium	References	[a]PHA accumulation (%)
A	Lee *et al.* (1994)	51
B	He *et al.* (1998)	62
C	Kim *et al.* (2000)	70
D	Yu *et al.* (2002)	80
E	Kojima *et al.* (2004)	17
F	Takagi *et al.* (2004)	69
G	Foster *et al.* (2005)	53
H	Zheng *et al.* (2005)	15
I	Full *et al.* (2006)	82
J	Berlanga *et al.* (2006)	68
K	Halet *et al.* (2007)	75
L	Ciesielski *et al.* (2006)	53
M	*E. coli* TISTR 527	-
N	Optimized medium	85

[a]Bacterial isolates when stained with 1% Nile blue A observed under fluorescent microscope and measured by Immage-ProPlus®, 6.0 Program

also investigated. The addition of 1 g L^{-1} of KH_2PO_4 and 3 g L^{-1} of Na_2HPO_4 in to optimized minimal medium was found to support the maximum accumulation of PHAs of bacteria isolated from cassava pulp when incubation at 30°C for 48 h. PHA granules in bacterial cells could be detected using specific cultural media and staining with lipophilic dyes such as Nile blue, Nile red or Sudan black B. However, fluorescence staining may cause flase-positive results or over explosions of PHA glanules in the bacterial cells. Precise and consistent detection methodology for bacterial PHA granules is still desired. TEM is one of the perfect detection method but there is no standard protocol avirable for these samples.

CONCLUSION

In this study, specimen preparation procedure for efficient observation of bacterial PHA granules under TEM was achieved. Fixative solution and dehydration series were especially developed for PHA which was lipid compound. The fixative solution composing of 5% glutaraldehyde, 1% OsO_4 and 0.2 M phosphate buffer was found to be suitable. Acetone at series (20, 40, 60, 80 and 100%) gave better results than ethanol in the dehydration step. This finding could enhance the detection of PHA granules using TEM.

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