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Growth Characteristics of Newly Isolated Xylose-Assimilating Bacterium and Accumulation of Green Plastic, Polyhydroxyalkanoate in the Genetic Engineered Strain

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Abstract A bacterial polyester, polyhydroxyalkanoate (PHA) is expected as "green plastic" because it is thermoplastic, flexible and biodegradable. In commercial production of PHA, the use of economic and ecological substrate like biomass is necessary. Hemicellulose, which is mainly composed of xylose and other monosaccharides can be a candidate of substrate for production of PHA. However, xylose is not easily utilized by microorganisms. We isolated a bacterium that grows at a high specific growth rate in xylose-mineral salts medium. The bacterium, which was named *Enterobacter* sp.TF, assimilates many kinds of sugar but it does not accumulate PHA. Hence, we made the genetic recombinants by introducing the genes for biosynthesis of homopolyester of D-3-hydroxybutyrate, PHB from *Ralstonia eutropha*, and the genes for biosynthesis of PHA copolyester from *Pseudomonas* sp.61-3. As a result, the recombinant introduced with the genes of *R.eutropha* accumulated PHB from xylose.

Keywords *Enterobacter*, Green plastic, PHB, Xylose

1. Background

Xylose is one of main component sugars of hemicellulose. For utilization of lignocellulosic biomass, it is important to convert xylose to useful compounds efficiently and cost-effectively. However, xylose is not easily utilized by microorganisms and the variety of fermentation products from xylose is small. It is expected to enlarge the variety of fermentation products by using newly isolated or engineered microorganisms. Poly(D-3-hydroxyalkanoate) (PHA) is the polyester accumulated in bacterial cells under imbalanced nutritional condition like nitrogen limitation. PHAs are expected as raw material for manufacturing of green plastic because they are thermoplastic, flexible and biodegradable. Poly(D-3-hydroxybutyrate) (PHB) was the first reported and well-known PHA. However, PHB is inferior in flexibility, then it is difficult to mold and fragile to stress of physical force. Therefore, the copolyester which is composed of both D-3-hydroxybutyrate and other types of hydroxyalkanoates are expected. In most of the researches for production of copolyester PHA, fatty acid, plant oil and hexose like glucose or fructose have been used as the substrate [1-11].

In this study, we investigated the fermentation characteristics of our newly isolated xylose-utilizing bacterium and the production of polyester from xylose in the genetically engineered strain.

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2. Methods

Isolation and identitication of xylose-assimilating bacterium

The water sampled from Onga River, which is flowing in the north area of Kyushu island, Japan, was spread on the mineral salts agar plate containing xylose as the sole carbon source and then it was incubated for 7 days at 30°C. Xylose-mineral salts agar medium was composed of $(NH_4)_2SO_4$ 2.0g, $MgSO_4 \cdot 7H_2O$ 0.2g, KH_2PO_4 1.0g, xylose 20g, agar 15g and trace elements solution 0.1mL per 1 L of distilled water. The composition of the trace elements solution was $FeSO_4 \cdot 7H_2O$ 16.0g, $CrCl_3 \cdot 6H_2O$ 0.13g,

$NiCl_3 \cdot 6H_2O$ 0.18g, sodium citrate dihydrate 15.6g, $CuSO_4 \cdot 5H_2O$ 0.1g, $CoCl_2 \cdot 6H_2O$ 15.6g in 100mL of 1M HCl. pH of the culture medium was adjusted to 6.8. After several trials, one bacterial colony was obtained.

Morphological and physiological tests were carried out for identification of the isolated bacterium. Homology for nucleotide sequence of the 16S rRNA gene against other bacteria was also investigated according to the scheme that we reported previously [12].

Culture test

Growth characteristics of wild strain and the genetically engineered strains of our isolated bacterium were investigated by flask culture and pH-controlled batch culture. pH-controlled batch culture experiment was carried out using a glass jar fermenter (total volume 1000mL; working volume 600mL) equipped with a pH controller (PHC-2201, Biott Co., Ltd., Tokyo) connected to a tube pump for feeding 4% ammonia water. Aseptic air was supplied through a sterile filter (i.d 0.2μm) at a flow rate of 0.5vvm to the fermenter during cultivation. The agitation speed was kept at 1200rpm. As the fermentation proceeded, the vigorous foaming of culture liquid occurred in the fermenter. Hence, a defoaming agent (Einol, Biott Co. Ltd., Tokyo) was periodically added to prevent the culture liquid overflowing from the fermenter.

Cell growth was monitored by measuring the optical density at 600nm (OD_{600}) of the culture liquid. The polyester accumulated in the cells was determined according to the method using gas chromatography [13]. The lyophilized cells, a mixture of 2mL of methanol acidified with 3% (w/v) H_2SO_4 and 2mL of chloroform were added into a screw cap vial and then it was heated at 100°C for 3.5h for degradation of the polyester, and esterification of hydroxyalkanoic acid and methanol. After cooling, 1mL of H_2O was added into a vial then the suspension was shaken well for 10min. After two phases were allowed to separate, the organic phase containing the methyl ester was applied to gas chromatography.

DNA manipulation and plasmid construction

In this study, three types of recombinant plasmids were introduced into *Enterobacter* sp.TF, respectively:- (i) The plasmid pRKmKSc-C1GAB was constructed to introduce the following genes for biosynthesis of PHA: β-ketothiorase gene (*phbA*) and acetoacetyl-CoA reductase gene (*phbB*) from *R.eutropha* to provide D-3-hydroxybutyrate, D-3-hydroxyacyl-acyl carrier protein (ACP)-CoA transferase gene ($phaG_{Ps}$) from *Pseudomonas* sp. 61-3 to provide various D-3-hydroxyalkanoates, PHA synthase gene ($phaC1_{Ps}$) having broad substrate specificities from *Pseudomonas* sp. 61-3 to polymerize various D-3-hydroxyalkanoates, and *lac* promotor. (ii) The plasmid pRKmKSc-C1G was constructed by eliminating *phbA* and *phbB* from the plasmid pRKmKSc-C1GAB. (iii) The plasmid pRKmBB-phbCAB, which is containing the genes for biosynthesis of PHB from *R.eutropha* ($phbCAB_{Re}$), was also used. All the plasmids used in this study are shown in Table 1.

Each of recombinant plasmid was introduced to *Enterobacter* sp.TF by conjugal transfer via *E.coli* S17-1. To isolate the colony harboring the plasmid, kanamycin ($50mg \cdot L^{-1}$) was added to xylose-mineral salts agar plate medium. All the DNA manipulations including the isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonucleases, agarose gel electrophoresis and transformation of *E. coli* S17-1 were performed by standard procedures [14].

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Enterobacter sp.TF	Wild type	This study
E. coli S17-1	*recA* and *tra* genes of plasmid RP4 integrated into the chromosome; auxotrophic for proline and thiamine	[15]
pBluescript II KS^+	Ap^r, *lacPOZ* T7 and T3 promoter	Stratagene
pBBR1MCS-2	Km^r, broad host range, *lac POZ'*	[16]
pJRD215	Cosmid; Km^r Sm^r RSF1010 replicon; Mob^+	[17]
pJBB49-*phb*	pJRD215 derivative; phb_{Re} promoter, $phaC1_{Ps}$, $phbAB_{Re}$	[18]
pBSKB-C1AB	pBluescript II KS^+ derivative; *lac* promoter, $phaC1_{Ps}$, $phbAB_{Re}$	This study
pBSHE9	pBluescript II KS^+ derivative; *lac* promoter, $phaG_{Ps}$	[13]
pRKmKSc-C1AB	pBBR1MCS-2 derivative; *lac* promoter, $phaC1_{Ps}$, $phbAB_{Re}$	[13]
pRKmHS32	pBBR1MCS-2 derivative; P_{Ps} promoter (*pha* promoter), $phaC1_{Ps}$, $phaG_{Ps}$	[19]
pBSHE9	pBluescript II KS^+ derivative; containing *phaG* of *Pseudomonas* sp. 61-3	[13]
pRKmKSc-C1GAB	pBBR1MCS-2 derivative; *lac* promoter, $phaC1_{Ps}$, $phaG_{Ps}$, $phbAB_{Re}$	[13]
pRKmKSc-C1G	pBBR1MCS-2 derivative; *lac* promoter, $phaC1_{Ps}$, $phaG_{Ps}$	[13]
pRKmBB-phbCAB	pBBR1MCS-2 derivative; P_{Re} promoter (*phb* promoter), $phbCAB_{Re}$	This study

3. Results

Identification of the isolated bacterium and assimilation of carbon source

The bacterium, which was isolated from Onga River with xylose-mineral salts agar plate, was Gram negative, straight rod (1.0–1.5μm), motile and facultatively anaerobic. Hydrolysis of gelatin and urease was also observed.

A scanning electron microscope image of the isolated bacterium is shown in Fig.1.

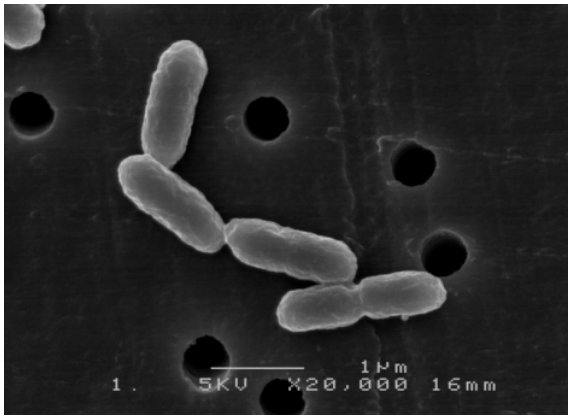

Figure 1. SEM image of isolated xylose assimilating bacterium

The bacterium assimilated broad kinds of monosaccharaides and disaccharides in mineral salts medium (Table 1). That is beneficial characteristic for utilization of hydrolysate of biomass composed of polysaccharides.

The optimum growth temperature was 35°C and the optimum pH was 6.3.

The sequence of the 16S rRNA gene of the isolated bacterium showed 98% identities to *Enterobacter* sp. CCBAU15488, *E.cloacae*, *E.agglomerans* and other strains of *Enterobacter* species. Then, we name our strain *Enterobacter* sp. TF.

The result of growth test for *Enterobacter* sp.TF on various kinds of carbon source is shown in Table 2.

Cell growth of *Enterobacter* sp. strain TF on xylose

Cell growth of *Enterobacter* sp. TF was investigated by flask culture with shaking at 170 rpm using $(NH_4)_2SO_4$ and KNO_3 with different concentrations as the nitrogen source. Xylose concentration was prepared to 20g·L^{-1}. The result is shown in Fig.2. Cell growth was superior in the cultures using $(NH_4)_2SO_4$ than KNO_3 and the growth rate was the fastest at $(NH_4)_2SO_4$ 2.0g · L^{-1} among all the tested conditions. However, the highest cell growth in all the culture tests was obtained when using $(NH_4)_2SO_4$ 2.0g·L^{-1}. Maximum cell growth in each culture test increased as the concentration of nitrogen source in culture medium increased. It was thought that cessation of cell growth was due to exhaustion of nitrogen source in culture liquid.

Table 2. Growth of *Enterobacter* sp.TF on various kinds of carbon source

D-Galactose	+	L-Arabinose	+	Trehalose	+	Cellulose	−
D-Glucose	+	Cellobiose	+	Erythritol	+	Acetate	Weak
D-Fructose	+	Lactose	+	Glycerol	+	Citrate	+
D-Mannose	+	Maltose	+	Mannitol	+	Lactate	+
D-Ribose	+	Melibiose	+	Xylitol	+	Propionate	Weak
D-Xylose	+	Sucrose	+	Starch	+		

Each carbon source was tested by adding 5g/L in the culture medium.

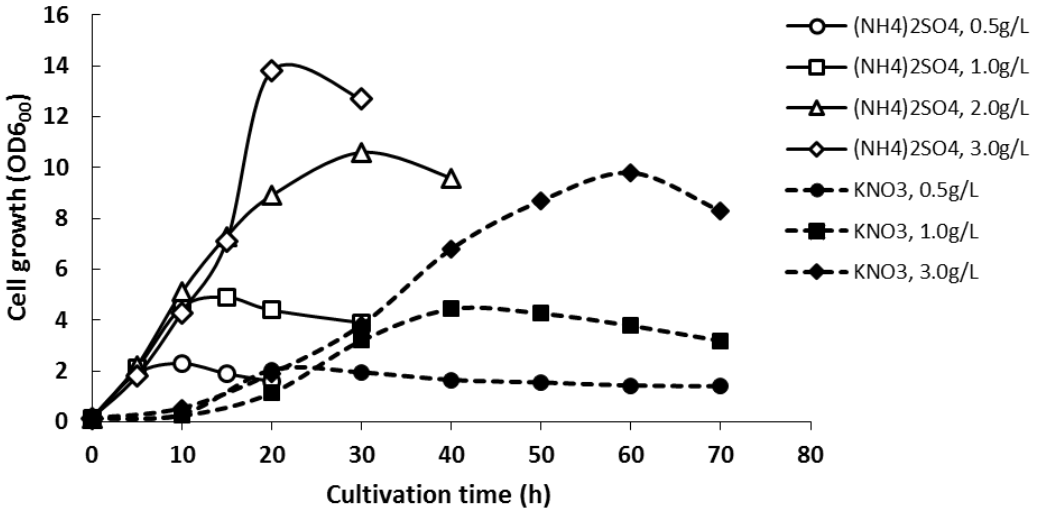

Figure 2. Cell growth of *Enterobacter* sp. TF in flask culture using $(NH_4)_2SO_4$ or KNO_3 with different concentrations as nitrogen source. Initial xylose concentration was 20g•L^{-1}

Effect of xylose concentration on the cell growth of strain TF was also investigated (Fig.3). Strain TF could grow at relatively high xylose concentration of 70g・L^{-1} and the growth rate became faster as the xylose concentration decreased. In flask culture, the decrease in culture pH was inevitable, which inhibited the cell growth (the data is not shown). The cessation of cell growth was due to the exhaustion of nitrogen source and the decrease in culture pH.

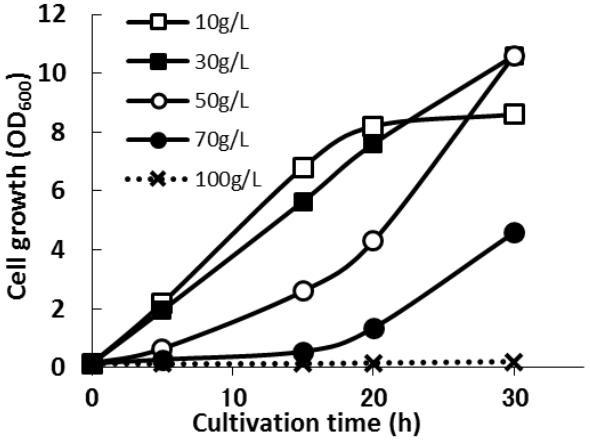

Figure 3. Cell growth of *Enterobacter* sp. TF in flask culture using culture medium with different xylose concentraions

Hence, pH-controlled culture test was carried out in batch wise using a jar fermentation system. pH was automatically maintained at 6.3 during cultivation by feeding 4% ammonia water as neutralizer and nitrogen source. The fermentation time course is shown in Fig.4. The initial concentration of xylose and $(NH_4)_2SO_4$ was 50g・L^{-1} and 2.0g・L^{-1}, respectively. The cell concentration increased to the 23.2 in OD_{600} after 25h of cultivation. The growth ceased due to the exhaustion of xylose in culture liquid. The specific growth rate was 0.70h^{-1}.

To investigate the accumulation of PHA in the strain TF, the cells grown in nitrogen-limited culture medium ($(NH_4)_2SO_4$ 0.5g/L) were harvested by centrifugation and then the lyophilized cells were treated for methanolysis. The esterified hydroxyalkanoic acid was analyzed by gas chromatography. The content of PHA in the cells (wt%) and the monomer composition were estimated. As a result, accumulation of polyester was not observed in the wild strain of *Enterobacter* sp.TF.

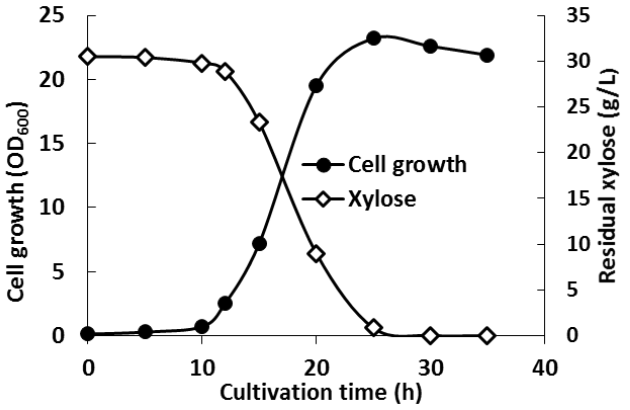

Figure 4. Fermentation time course of *Enterobacter* sp. in pH-cntrolled batch culture with feeding 4% ammonia water. pH was maintained at 6.3

PHA accumulation from xylose in recombinant of *Enterobacter* sp. strain TF

It was shown that strain TF does not produce any polyesters in the cell from xylose. However, the specific growth rate was much higher than that of *Ralstonia eutropha* on fructose [20]. *R.eutropha* is a "handy" organism [21] and the most frequently used for the production of PHA due to the high levels of polyester accumulation, and the availability of genetic tools and ease of genetic manipulation. Therefore, *Enterobacter* sp. TF was used as the host for genetic engineering for biosynthesis of PHA from xylose. Three types of plasmids, pRKmKSc-C1GAB, pRKmKSc-C1G and pRKmBB-phbCAB were respectively introduced to the wild strain of *Enterobacter* sp. TF. The obtained three recombinant strains were grown in flask culture using xylose medium added with kanamycin (50mg・L^{-1}), respectively and then the accumulation of polyester in the cells was investigated (Table 3).

Table 3. Intracellular content of PHA produced from xylose in recombinants of *Enterobacter* sp.TF and the monomer unit composition

Strains/plasmid	PHA content (wt%)	PHA composition (mol%)				
		C_4 (3HB)	C_6 (3HHx)	C_8 (3HO)	C_{10} (3HD)	C_{12} (3HDD)
Enterobacter/pRKmKSc-C1G	0	0	0	0	0	0
Enterobacter/pRKmKSc-C1GAB	2.02	87.9	0	0	12.1	0
Enterobacter/pRKmBB-phbCAB	27.3	100	0	0	0	0

3HB, D-3-hydroxybutyrate; 3HHx, D-3-hydroxyhexanoate; 3HO, D-3-hydroxyoctanorate; 3HD, D-3-hydroxydecanoate; 3HDD, D-3-hydroxydodecanoate. The data columns which are described with "0" mean that GC analysis was carried out but the substance was not detected.

In the recombinant strain *Enterobacter*/pRKmKSc-C1G which is harboring *lac* promoter, $phaG_{Ps}$ and $phaC1_{Ps}$ from *Pseudomonas* sp.61-3, polyester was not detected. In the recombinant strain *Enterobacter*/pRKmKSc-C1GAB which is harboring *lac* promoter, $phaG_{Ps}$, $phaC1_{Ps}$ and *phb*AB from *R.eutropha*, the copolyester composed of 87.9mol% D-3-hydroxybutyrate and 12.1mol% D-3-hydroxydodecanoate was observed but the intracellular content in the cells was very low (2.02wt%). Hence, we made the third recombinant strain introduced with the plasmid pRKmBB-phbCAB and then we investigated the accumulation of the polyester. The plasmid pRKmBB-phbCAB was incorporated with PHB biosynthesis operon of *R. eutropha* that contains *phbAB*, PHB synthase gene from ($phbC_{Re}$) and native promoter (phb_{Re} promoter). As a result, the obtained recombinant *Enterobacter*/ pRKmBB-phbCAB accumulated PHB at much higher content in the cells than *Enterobacter*/pRKmKSc-C1GAB.

4. Discussion

The enzyme PhaG is a D-3-hydroxyacyl-acyl (3HA) carrier protein (ACP)-CoA transferase providing various D-3-hydroxyacyl-CoAs from fatty-acid biosynthetic pathway as the substrate of PHA. However, the accumulation of PHA or PHB in the two recombinant strains incorporated with the gene *phaG* was very poor. *Enterobacter*/pRKmKSc-C1G did not accumulate polyester in the cells while *Enterobacter*/pRKmKSc-C1GAB accumulated slight amount of poly(D-3-hydroxybutyrate-*co* -D-3-hydroxydecanoate). On the other hand, the *Enterobacter*/pRKmBB-phbCAB, which has no *phaG*, accumulated PHB in the cells over 20wt%. Recently, Wang et al. reported that the enzyme PhaG is not only functioning as 3HA carrier protein (ACP)-CoA transferase but also functioning as 3-hydroxyacyl-ACP thioesterase [5]. Therefore, in the two recombinants incorporated with *phaG*, D-3HA-CoA formed in the cells would be immediately converted to free D-3-hydroxyalkanoic acids, which caused the decrease in intracellular content of polyester. Actually, HPLC analysis for culture supernatant of the *Enterobacter*/ pRKmKSc-C1GAB indicated that several kinds of organic acids like pyruvate and L-malate are excreted from the cells and the amount is not small (the data is not shown). It is known that xylose is metabolized mainly via pentose phosphate (PP) pathway/glycolytic pathway or phosphoketolase (PK) pathway. In case of some lactic acid bacteria, the PK pathway yielding acetate, formate and ethanol, and the PP/glycolytic pathway converts xylose to L-lactate only. Furthermore, it is also reported that the change in the xylose concentration shifts the metabolism between the PK pathway and the PP/glycolytic pathway and pyruvate metabolism between cleavage to acetyl-CoA and formic acid by pyruvate-formate lyase and the reduction to L-lactate by lactate dehydrogenase, therefore the yield coefficient of lactate/xylose changes according the xylose concentration [22].

In recent years, many researchers have reported for microbial production of PHB and copolyester PHA from xylose or other pentose derived from hemicellulose, for instance, engineered *E.coli* [23] and yeast [24], mcl-PHA production from xylose and octanoic acid by engineered *Pseudomonas putida* KT2440 [25], screening of bacteria to produce PHB from xylose [26], PHA production from sugar maple hemicellulosic hydrolysate by *Burkholderia cepacia* [27], and PHA production from lignocellulosic materials [28]. In those reports, the yield, content and productivity of PHB or PHAs from xylose were still lower compared to those from glucose or organic acids.

To increase the content of PHA in the engineered *Enterobacter* sp.TF and the molar ratio of second unit, it is necessary to elucidate the metabolic pathway of xylose. It is also expected that the introduction of D-3-hydroxyacyl (3HA)-CoA ligase gene, which is available to provide mcl-3HA units efficiently to polymerization from fatty acid biosynthesis pathway, may improve the accumulation of copolyester.

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