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Characterization of *Burkholderia* sp. Y1 Isolated from Oil Polluted Soil

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Burkholderia sp. Y1 was isolated from oil polluted soil as a lipid-degrading bacterium. Strain Y1 degraded 83.1% of salad oil when it was cultivated in the basic medium containing 1% (w/v) of salad oil for 2 days at 35°C. When cultivated in medium containing 5 and 10% (w/v) of oil, strain Y1 showed lipid degradation rates of 30.2% and 15.4%, respectively, suggesting that strain Y1 could maximally assimilate 0.15 g/l salad oil. The lipid degradation was dependent on the initial cell density. Thin layer chromatography (TLC) analysis of culture supernatant indicated that strain Y1 first dehydrated triglycerides in salad oil in the degrading process and produced free fatty acids, diglycerides and monoglycerides, then gradually degraded residues. Also, gas chromatography (GC-FID) analysis indicated that strain Y1 was able to assimilate C16 to C20 of fatty acids in salad oil equally.

Key words: lipid degradation, *Burkholderia* sp., waste water treatment

1. Introduction

All facilities discharging waste water containing oils are required to be equipped with grease traps in Japan. It is estimated that an average of around 1.1 kg of oil is discharged in a year from one facility which discharges less than 50 m^3 of waste water per day[4]. The waste oil accumulated in grease traps has to be removed frequently, and it generally costs ten to thirty thousand yen per month. To reduce the cost of handling grease traps is a significant problem.

Many detailed researches have been reported concerning lipid-degrading bacteria such as *Acinetobacter*[17,19], *Pseudomonas*[21,5] or *Rhodococcus*[9]. Similarly, there have been several reports concerning the effectiveness of bacterial consortium[20,1]. However, most of them were conducted using media containing only less than 1% (w/v) oil, and it took a long time for microbes to degrade oil. At an actual site, a much higher oil density is accumulated and a more rapid treatment system is required. In this study, we isolated and characterized a bacterium able to degrade high concentrations of salad oil.

2. Material and methods

2.1. Reagents

Without some exception, all reagents were at ultra pure grade and were purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan and salad oil was purchased from a market.

2.2. Media

The stock solution used for suspending isolated microbes contained 8.0 g of NaCl, 2.0 g of KCl, 36.3 g of $Na_2HPO_4 \cdot 12H_2O$, 2.4 g of KH_2PO_4 and 200 ml of glycerol in one liter of sterilized water. This solution was adjusted to pH 8.0, then autoclaved at 121°C for 15 min. The basic medium used for enrichment culture and estimating lipid degradation rate contained 1.0 g of NaH_2PO_4, 2.0 g of K_2HPO_4, 2.0 g of $(NH_4)_2HPO_4$, 0.1 g of yeast extract and 0.2 g of $MgSO_4 \cdot 7H_2O$ in one liter of sterilized water, (pH 7.0)[12]. Standard Method Agar (SMA), Lactobacilli MRS Agar (MRSA) (Becton, Dickinson and Company Sparks, MD, USA) and Yeast Extract Glucose Chrolamphenicol Agar (YEGCA) (Becton) mixed with victoria blue dye base oil (VBO) were used for isolating lipid-degradation microbes. VBO was prepared following the procedure of Standard Method for the Count of Lipolytic Organisms[6].

2.3. Isolation of lipid degradation microbes

Suspensions of (1% (v/v)) soil or liquid samples collected from oil polluted sites were inoculated to 10 ml of basic medium containing 10% (w/v) of salad oil and incubated at

35°C for 7 days with 100 rpm agitation. The culture showing the highest lipid degradation rate was chosen for an isolating source. The sample suspension was subjected to enrichment cultivation again under the same conditions for 2 days, and the culture broth was spread on SMA, MRSA and YEGCA supplemented with VBO for single colony isolation. Each isolated strain was cultured on SMA without VBO and colonies were suspended in the basic medium at optical density at 660 nm (OD_{660}) to 1.0 (approximately 10^9 CFU/ml), then incubated with 1% (w/v) of salad oil at 35°C for 7 days with 100 rpm agitation to estimate the lipid degradation rate.

2.4. Measurement of lipid degradation

The lipid degradation rate was estimated by the hexane extraction method[5] with a slight modification. Briefly, the culture broth was transferred to a 50 ml centrifuge tube. The flask was washed three times with 5.0 ml of hexane to completely remove salad oil and added to transferred culture broth. After adding 1.0 g of sodium chloride, suspension was shaken for 5 min and ultrasonically treated for 10 min at 50°C, then centrifuged 1,500×*g* for 10 min. The hexane layer was transferred to a new tube and the volume was made up to 30 ml with hexane. One milliliter of this hexane solution was used for TLC and GC-FID, and the remaining solution was transferred to an aluminum cup. After evaporation on a hot plate for 10 min, the hexane extracts were heated to dry at 80°C for 30 min and cooled down for 30 min in a desiccator. The residues were weighed to estimate the lipid degradation rate.

2.5. Identification of strain Y1

Strain Y1 was identified by morphological and biochemical tests[16] and 16S rDNA sequence analysis. DNA was extracted from strain Y1 as previously described[13] and its 16S rDNA was amplified by polymerase chain reaction (PCR) using primers 9F and 1541R (Table 1). The PCR mixture contained 30 ng of genomic DNA, 3.2 pmol of each primer, 1 fold of buffer solution, 25 mmol of dNTPs and 5 units of Taq polymerase (TaKaRa Bio Inc, Shiga, Japan). PCR amplification was carried out at 94°C for 4 min, then 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and finally 72°C for 15 min[14] and the amplified DNA was purified by phenol-chroloform method. Sequencing reaction was carried out with a Big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's specifications by using sequencing primers 9F, 341F, and 515F. Sequencing was automatically performed on an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems). The sequences were compared with the compilation of 16S rDNA genes available in the database (DDBJ, EMBL and GenBank).

2.6. TLC analysis

The hexane extracts were analyzed by TLC using TLC plastic sheets of silica gel 60 F_{254} (Merck, Darmstadt, Germany), with a mobile phase of hexane, diethyl ether and acetic acid (80:30:1, v/v/v). Mono-, Di- and Triglyceride Mix (SUPELCO) was spotted for the standard. The spots of lipids and residual products were detected using sulfuric acid steam[2].

2.7. GC-FID analysis

The hexane extracts were esterified by the Boron Trifluoride-Methanol Method[7]. Esterified substrates were dissolved in dichloromethane up to 1 ml. n-Dodecane (C12) was added in each sample as the internal standard. One microliter of the suspension was analyzed with a gas chromatograph (Auto-System XL GC, Perkin Elmer Corporation, CT 06859-0010, USA) equipped with a flame ionization detector (FID) and a capillary column (DB-23, length 30 m, I.D. 0.25 mm, film thickness 0.1 µm, J&W Scientific, USA). The carrier gas was helium, and injector and detector temperatures were set at 260°C. The column temperature was first held at 60°C for 2 min, then raised to 140°C at the rate of 30°C min^{-1}, continuously raised to 240°C at the rate of 2°C min^{-1}, and finally held for 10 min at 240°C. Oil contents were sequenced by comparing to Supelco 37 Component FAME Mix (FAME 37) (SUPELCO, Bellefonte, PA).

3. Result and Discussion

3.1. Isolation and identification of lipid degrading microbes

Oil contaminated soil and water samples were collected from 60 sites. In the first screening, over 10% lipid degradation rate was observed with 10 samples. Eighty strains were isolated from the sample exhibiting the highest lipid degradation rate in the first screening. Restriction fragment length polymorphism of 16S rDNA revealed that isolates were classified into 4 different groups. Among them, strain Y1 showed the highest lipid degradation rate, approximately 80% after cultivating with the 1% (w/v) of salad oil in the basic medium at 35°C for 2 days. The morphological and biochemical tests showed that strain Y1 was a gram nega-

Table 1. PCR and sequencing primers used in this study.

Primer No.	*E. coli* numbering	Base sequence	Reference
9F	9–28	5'-GAGTTTGATCCTGGCTCAG-3'	11
341F	341–357	5'-CCTACGGGIGGCIGCA-3'	8
515F	515–534	5'-GTGCCAGCMGCCGCGGTAA-3'	15
1541R	1521–1541	5'-AAGGAGGTGATCCANCCRCA-3'	18

tive, catalase reaction positive, oxidase reaction positive and small rod shape bacteria. Strain Y1 grew and degraded salad oil well at temperatures from 28°C to 35°C. Optimal temperature for oil degradation was 30°C. Strain Y1 also grew on YEGCA, indicating that strain Y1 is tolerant to chloramphenicol (Table 2).

The carbon source assimilating pattern of strain Y1 was analyzed by BioLog automated microbial identification/characterization system (Biolog, Inc., Hayward, CA, USA) for the gram-negative microplates in aerobic conditions[10]. Strain Y1 was able to metabolize 62 of 95 substrates in the panel, indicating that the strain Y1 is most closely related to *Burkholderia pyrrocinia* with 60.8% similarity (data not shown). In addition, 16S rDNA analysis was performed in order to reconfirm the identification of strain Y1 as *B. pyrrocinia*. A 1031-bp DNA fragment containing the 16S rRNA gene was amplified by PCR and sequenced. Similarity search with the BLAST program found that the 16S rRNA gene of strain Y1 was 100% identical to that of *Burkholderia cepacia* strain ESR63 (EF602558). Although tere is discrepancy between Biolog and 16S rRNA analysis, both analysis classified strain Y1 to *Burkholderia*, we identified strain Y1 as *Burkholderia* sp. strain Y1 (EMBL/GenBank/DDBJ accession Number AB376079).

3.2. Characterization of lipid degradation ability of strain Y1

Further experiments were performed to investigate the ability of strain Y1 to degrade salad oil. First, effects of oil concentrations on the degradation rate of strain Y1 were investigated. Strain Y1 was cultivated in the basic medium containing 1%, 5% and 10% (w/v) salad oil at 35°C for 2 days. The initial cell density (OD_{660}) was adjusted to 1.0. The lipid degradation rate at 1, 5 and 10% (w/v) were 83.1%, 30.2% and 15.4% (0.083, 0.151 and 0.154 g/l oil density), respectively (Fig. 1). This indicates that strain Y1 is able to maximally degrade 1.5% (w/v) of oil. Since the salad oil physically attached to the flask wall during cultivation, the lipid degradation rate on the condition of 1% (w/v) oil might not reach to 100%. Then, effects of the initial cell density on the oil degradation were investigated. The initial cell density (OD_{660}) was adjusted to 0.01, 0.1 and 1.0 and the lipid degradation rate at 1% (w/v) of salad oil was determined after cultivation for 5 days. The highest degradation rate was observed when the initial OD_{660} was 1.0 (Fig. 2). In that cultivation, the lipid degradation reached 77.4% on 1 day after the start of cultivation and gradually increased to 90% on 2 days. The degradation rates with the initial OD_{660} of 0.01 and 0.1 were lower than that with the initial OD_{660} of 1.0 and the maximum degradation of 80–87% was observed on 4 days after the start of cultivation. Thus, the lipid degradation by strain Y1 is dependent on the initial cell density.

Table 2. Characteristics of strain Y1.

Gram stain	–
Bacterial shape	Rod
Colony color (on SMA without VBO)	Dark cream
Catalase reaction	+
Oxytase reaction	+
Growth on:	
SMA aerobic	++
anaerobic	+
MRSA anaerobic	–
YEGCA aerobic	+–
anaerobic	+
Growth at:	
28°C	+
30°C	++
35°C	+
37°C	+

++, strongly positive; +, positive; +–, weakly positive and –, negative.

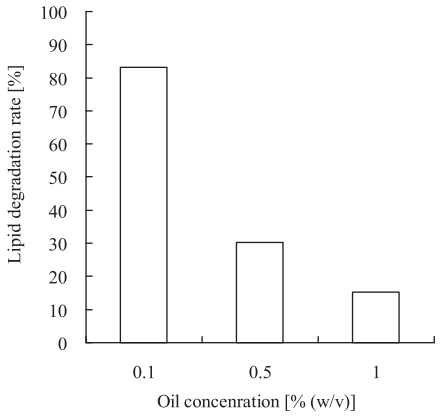

Fig. 1. Effects of oil concentrations on lipid degradation by strain Y1.
Strain Y1 was cultivated in the basic medium containing 1%, 5% and 10% (w/v) salad oil at 35°C for 2 days. The initial cell density (OD_{660}) was adjusted to 1.0.

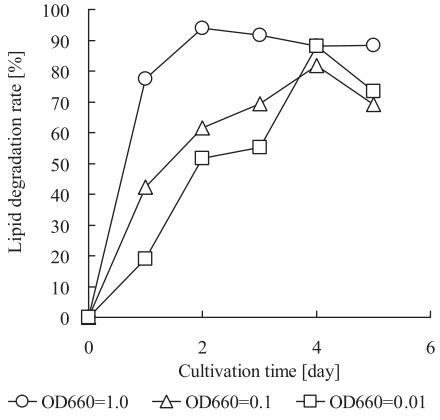

Fig. 2. Effects of the initial cell density on lipid degradation by strain Y1.
The initial cell density (OD_{660}) was adjusted to 0.01 (squares), 0.1 (triangles) and 1.0 (circles) and the lipid degradation rate at 1% (w/v) of salad oil was determined after cultivation for 5 days.

3.3. Mechanism of lipid degradation by strain Y1

We then characterized the salad oil degradation process of strain Y1. Strain Y1 (the initial OD_{660}=1.0) was cultivated in the basic medium with 1% (w/v) salad oil at 35°C. Culture broth was sampled everyday and its supernatants were subjected to TLC and GC analysis. Strain Y1 was able to dehydrate triglyceride in salad oil quite well, and produced free fatty acids, diglycerides and monoglycerides on the first day (Fig. 3). After dehydration of triglyceride, strain Y1 assimilated all residual oil components. Culture broth on the fifth day was analyzed by GC-FID. The salad oil used in this study contained C12 to C20 fatty acids. The GC-FID showed that strain Y1 was able to degrade about 80% of all components in salad oil during 5 day cultivation (Fig. 4). The area calculation found 89.0% total reduction rate, and this is strongly related to the hexane-extracted lipid degradation rate of 88.3%.

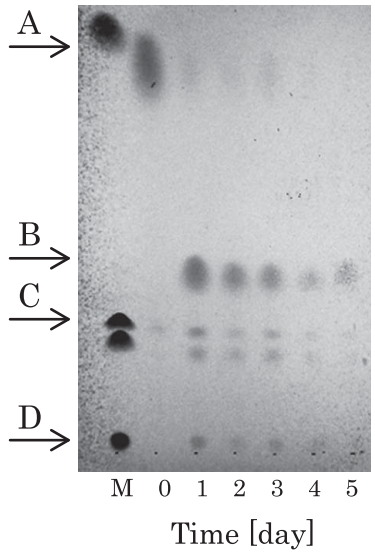

Fig. 3. TLC analysis of culture supernatants of strain Y1.
Strain Y1 (OD_{660}=1.0) was cultivated in the basic medium with 1% (w/v) salad oil at 35°C.
Laine M, Fame37; arrow A, triglyceride; arrow B, diglyceride; arrow C, free fatty acid; arrow D, monoglyceride.

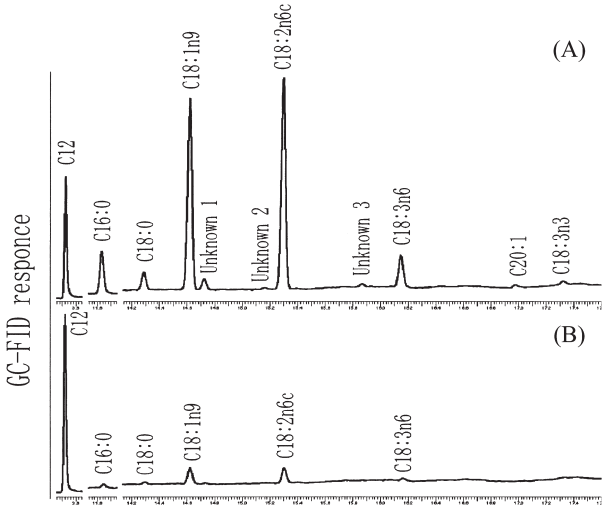

Fig. 4. GC-FID analysis of nonculture (A) and culture (B) supernatants of strain Y1.
Strain Y1 (OD_{660}=1.0) was cultivated in the basic medium with 1% (w/v) salad oil at 35°C. Culture broth was sampled after 5 days cultivation and its culture supernatants were esterified for GC analysis equipped with flame ionized detector. *n*-Dodecane was used as the internal standard.

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