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(54) Title: BACTERIAL CONSORTIUM EBC1000 AND A METHOD USING THE BACTERIAL CONSORTIUM EBC1000 FOR REMEDIYING BIOLOGICALLY RECALCITRANT TOXIC CHEMICALS CONTAINED IN INDUSTRIAL WASTEWATER, WASTE MATERIALS AND SOILS

(57) Abstract: The present invention relates to a novel bacterial consortium EBC1000 (KCTC 0652 BP) and a method for remedying biologically a chlorine compound and recalcitrant toxic chemicals contained in industrial wastewater, waste materials and soils using the bacterial consortium EBC1000. The novel bacterial consortium EBC1000(KCTC 0652 BP) is composed of aerobic bacterium easy to culture and useful for decomposing a chlorine compound and waste acidic or alkaline recalcitrant toxic chemicals of high concentration contained in the industrial waste materials, such as IPA, MC, DMA, AN, SHS, BD, Tamol-SN, EDTA, FES, TDDM, PMH, DEHA, MeOH, NaOH and CH<sub>3</sub>CN, thus preventing and recovering environmental pollutions.

**BACTERIAL CONSORTIUM EBC1000 AND A METHOD USING THE  
BACTERIAL CONSORTIUM EBC1000  
FOR REMEDYING BIOLOGICALLY RECALCITRANT TOXIC  
CHEMICALS CONTAINED IN INDUSTRIAL WASTEWATER, WASTE  
5 MATERIALS AND SOILS**

**TECHNICAL FIELD**

The present invention relates to a novel bacterial consortium EBC1000 and a  
10 method for remedying biologically recalcitrant toxic chemicals contained in industrial  
wastewater, waste materials and soils using the bacterial consortium EBC1000. More  
particularly, the invention relates to a novel bacterial consortium EBC1000 capable of  
remarkably decomposing a chlorine compound of high concentration and waste acidic  
or alkaline recalcitrant toxic chemicals difficult to be degraded that are isolated from  
15 waste soil and water, the recalcitrant toxic chemicals having a chemical oxygen  
demand (COD<sub>Mn</sub>) of 20,000 to 100,000 ppm and comprising chemicals, such as  
isopropyl alcohol(IPA), methylene chloride(MC), dimethylamine(DMA),  
acrylonitrile(AN), hydrosulfite(SHS), butadiene(BD), sodium alkylaryl naphthalene  
sulfonate(Tamol-SN), tetra sodium ethylene diamine tetra acetate dihydrate(EDTA),  
20 ferrous sulfate heptahydrate(FES), tert-dodecyl mercaptan(TDDM), paramethane  
hydroperoxide(PMH), N-diethyl hydroxyl amine(DEHA), methanol(MeOH), NaOH  
and CH<sub>3</sub>CN.

**BACKGROUND ART**

25 Environmental contaminations caused by recalcitrant toxic substances causes

degradation of food stuffs for human related to the food chain in the biosphere, prevalence of infectious diseases due to deteriorated immunity, spread of chronic diseases and energy exhaustion. Once the ecosphere is radically destroyed, it may take several hundred millions of years for recovery of the environment of ecosphere.

5           There has been an attempt to remedy recalcitrant toxic substances contained in the industrial wastewater or noxious waste materials dumped into the sea normally, using physical and chemical methods such as incineration, landfill, chemicals, electrolysis, membrane separation or the like. However, these conventional methods also involve economical and environmental problems. The most efficient and safe  
10 method that meets requirements in every aspect of environmentology, sanitation, ecology and economy is the biological treatment method. Many studies have been made on the biological methods, for example, using the natural ecosystem such as silt at an estuary. However, these methods could not be a solution of treating a great mass of industrial waste materials.

15

#### **DISCLOSURE OF THE INVENTION**

It is, therefore, an object of the present invention to provide bioremediation to remedy biologically recalcitrant toxic chemicals contaminating industrial wastewater, waste materials and soils, etc., using a trace of useful specific bacterial consortium that  
20 exists in the ecological system.

The present invention is directed to a novel bacterial consortium EBC1000 and a method for remedying biologically recalcitrant toxic chemicals and chlorine compounds contained in industrial wastewater, waste materials, soils, or the like using the bacterial consortium EBC1000. More particularly, the invention is directed to a

novel bacterial consortium EBC1000 capable of remarkably degrading chlorine compounds of high concentration and waste acidic or waste alkaline recalcitrant toxic chemicals that are isolated from soil and wastewater having COD<sub>Mn</sub> of 20,000 to 100,000 ppm, such as contained in the medical industrial wastewater to be dumped  
5 into the sea, e.g., isopropyl alcohol, methylene chloride, NaOH, Na<sub>2</sub>SO<sub>4</sub>, dimethylamine or methanol, or as contained in the petroleum industrial wastewater to be dumped into the sea, e.g., sodium hydrosulfite, butadiene, sodium alkylaryl naphthalene sulfonate, acrylonitrile, tetra sodium ethylene diamine tetra acetate dihydrate, ferrous sulfate heptahydrate, tert-dodecyl mercaptan, paramethane  
10 hydroperoxide, N-diethyl hydroxy amine, and CH<sub>3</sub>CN.

The inventor isolated a novel bacterial consortium EBC1000 (KCTC 0652 BP) having a characteristic of rapidly degrading recalcitrant waste materials of high concentration dumped into the sea as well as chlorine compounds, from the soils and wastewater collected from the Ulsan industrial complex and the petroleum chemical  
15 industrial complex in Korea. The bacterial consortium EBC1000 consists of nine strains, each having the ability of degrading recalcitrant waste materials. It was found that the bacterial consortium EBC1000 degrades waste acidic or waste alkaline recalcitrant waste materials having the COD<sub>Mn</sub> of 20,000 to 100,000 ppm contained in the medical and petroleum industrial wastewaters to be dumped into the sea by 80%  
20 within 7 days, 90% within 10 days and at least 80 to 90% within 240 hours in an aerobic manner. Examples of the waste acidic or alkaline recalcitrant waste materials contained in the medical industrial wastewater of e.g., pH 3 or 14 include isopropyl alcohol (IPA, 10,000 to 20,000 ppm), methylene chloride (MC, 100 to 7,000 ppm), NaOH (changeable), Na<sub>2</sub>SO<sub>4</sub> (changeable), dimethylamine (DMA, 70 to 2,500 ppm),

methanol (MeOH, 27,000 to 54,000 ppm), organic matters and antibiotic residues(40,000 to 100,000ppm) and Cl<sup>-</sup>(4,000 to 33,000ppm). Examples of the waste acidic or alkaline recalcitrant waste materials contained in the petroleum industrial wastewater of e.g., pH 1.2, 3.0 or 5.0 include sodium hydrosulfite (SHS, (NaSO<sub>2</sub>)<sub>2</sub>, 30  
5 ppm), butadiene (BD,changeable), sodium alkylaryl naphthalene sulfonate (Tamol-SN, 4,000 to 20,000 ppm), acrylonitrile (AN,changeable), tetra sodium ethylene diamine tetra acetate dihydrate (EDTA, 20 to 200 ppm), ferrous sulfate heptahydrate (FES, 60 ppm), tert-dodecyl mercaptan (TDDM, 4,000 ppm), paramethane hydroperoxide (PMH, 400 ppm), N-diethyl hydroxyl amine (DEHA, 200 ppm), and CH<sub>3</sub>CN  
10 (changeable).

Accordingly, the present invention is to provide such a novel bacterial consortium EBC1000 capable of degrading recalcitrant toxic chemicals and chlorine compounds, and a method using the bacterial consortium EBC1000 in remedying biologically recalcitrant toxic chemicals and chlorine compounds contaminating  
15 industrial wastewater, waste materials and soils.

### **BEST MODE FOR CARRYING OUT THE INVENTION**

Now, a description will be made to isolation, identification and activity of the novel bacterial consortium EBC1000.

#### 20 1. Isolation of Novel Bacterial Consortium EBC1000

(1) Isolation of 40 strains from shaking culture of the samples.

The soil (1 g) and wastewater (10 ml) from the Ulsan industrial complex were inoculated in a waste acidic and alkaline waste liquid medium(prepared by dilution of K<sub>2</sub>HPO<sub>4</sub> (0.65 g), KH<sub>2</sub>PO<sub>4</sub> (0.17 g), MgSO<sub>4</sub> (0.1 g), NaNO<sub>3</sub> (0.5 g) and 10% waste

acidic and alkaline wastewater containing recalcitrant chemicals in medical and petroleum industrial waste liquid, in 1 liter of deionized water, pH 0 to 14) and then in a chlorine compound liquid medium(prepared by dissolving of  $K_2HPO_4$  (0.65 g),  $KH_2PO_4$  (0.17 g),  $MgSO_4$  (0.1 g),  $NaNO_3$  (0.5 g) and pentachlorophenol (PCP, 50 ppm) in 1 liter of deionized water, pH 7.2), followed by shaking culture at 20 to 30 °C for more than 5 days.

1 ml of the shaken culture was collected and inoculated in a waste acidic or waste alkaline solid medium (prepared by adding only 1.5% of agar to the waste acidic or alkaline liquid culture, pH 7.0) and then a chlorine compound solid medium(prepared by adding 20 mg of BTB and 1.5% of agar to the chlorine compound liquid medium), followed by incubation at 20 to 30 °C for 3 to 10 days.

The individual pure colonies isolated were further inoculated in the above-mentioned cultures and then shaking cultured, thus isolating 40 useful bacterial strains which have a different colony form formed respectively and the same colony after successive transfer culture.

(2) Isolation of 9 competent strains after the bacterium isolated in step (1) are cultured in the medium of an increased concentration of the recalcitrant wastewater.

The bacterium isolated in step (1) were in order and individually inoculated in a minimal medium containing 50% of the crude wastewater and 80% of wastewater containing the recalcitrant chemicals of the medical and petroleum wastewater and then a minimal medium containing 500 ppm of the pentachlorophenol compound and repeated to the same procedures of step (1), thus isolating nine competent strains viable in the wastewater of higher concentration of the recalcitrant chemicals.

The nine bacterial strains, which constitute a single bacterial consortium,

named "EBC1000", are designated as EBC100, EBC101, EBC103, EBC104, EBC105, EBC106, EBC107, EBC108 and EBC109.

The novel bacterial consortium EBC1000 was deposited in the Korean Collection for Type Cultures in the Korea Research Institute of Bioscience and  
5 Biotechnology as the deposition No. KCTC 0652 BP on August 12, 1999.

## 2. Optimum Conditions for Growth of Bacterial Consortium EBC1000

The bacterial consortium EBC1000 had the best growth in the Luria-Bertani nutrient medium (containing 10 g of bacto-tryptone, 5 g of bacto-yeast extract and 10 g of NaCl in 1 liter of deionized water) at pH 5 to 8 and 25 to 35 °C with shaking at 50  
10 to 100 rpm/min for 24 to 48 hours. The bacterial consortium EBC1000 was also well grown in the successive transfer culture under the same conditions.

## 3. Identification of Bacterial Consortium EBC1000

The individual strains constituting the bacterial consortium EBC1000, designated as EBC100, EBC101, EBC103, EBC104, EBC105, EBC106, EBC107,  
15 EBC108 and EBC109 are a mixture of Gram negative and positive bacterium and have unique shape and size. After culture for 24 hours in the Luria-Bertani medium, EBC100 forms a 1mm-diameter circular colony, EBC101 a large about 2mm-diameter circular colony, EBC103 a thick about 2mm-diameter circular colony, EBC104 and EBC105 a small about 0.5mm-diameter circular colony, EBC106 a thick about 3mm-  
20 diameter colony, EBC107 a about 1.2mm-diameter brownish colony, EBC108 a about 1mm-diameter yellowish colony, and EBC109 a about 2-diameter double circle. These nine strains are aerobic and air permeable bacterium. EBC100, EBC101, EBC103, EBC106, EBC107 and EBC108 have high viability in the strong acidic condition of pH 3 to 4 and in the strong alkaline condition of pH 9 to 11. EBC104, EBC105 and

EBC109 are slow in growth and EBC100, EBC104, EBC105 and EBC109 show mobility.

For the genera of the individual constituent strains of the bacterial consortium EBC1000 included EBC100, EBC101 and EBC103 are the strains of the genus  
5 *Klebsiella*, EBC105 *Providencia*, EBC104 and EBC109 *Escherichia*, EBC106 *Bacillus*, EBC107 Gram-negative bacteria and EBC108 Gram-positive Bacteria.

The characteristics of the individual strains are presented in Tables 1 and 2.

[Table 1a]

Characteristic	EBC 100	EBC 101	EBC 103
Gram Strains	-	-	-
Catalase	+		
Oxidase	-		
Urease	+		
Citruse Utilization	+		
Glucose Utilization	+		
V-P Test	+		
Lysine Decarboxylase	+		
Ornithine Decarboxylase	-		

10

[Table 1b]

Characteristic	EBC 100	EBC 101	EBC 103
Inositol	+		+
Arabinose	+	+	+



Mannitol	+	+	+
Rhamnose	+	+	+
Glucose	+	+	+
Sorbitol	+	+	+
$\alpha$ -cyclodextrin	-	-	-
Dextrin	+	+	-
Glycogen			-
Adonitol	+	+	
D-Arabitol		+	+
Cellobiose		+	+
D-Fructose	+	+	+
L-Fucose	+	+	+
D-Galactose	+	+	+
$\alpha$ -Lactose			-
Maltose		+	+
D-Raffinose		+	+
D-Trehalose	+	+	+
Methyl-pyruvate	-	+	+
Citric Acid	+	+	+
Formic Acid			+
Malonic Acid	-	-	
Succinic Acid			+
D-Alanine			+

L-Alanine	+		+
L-Glutamic Acid		+	+
L-Serine	+	+	+
D, L-Lactic Acid	+	+	+
Mobility	+	+	+
D-Mannose	+	+	+

[Table 2]

Characteristic	EBC 104	EBC 105	EBC 109
Gram Strains	-	-	-
ONPG	+	-	+
Arginine	-	-	-
Lysine	+	-	-
Ornithine	-	-	-
Sodium Citrate	-	-	-
Sodium Thiosulfate	+	-	+
Urea	-	+	-
Tryptophane	-	+	-
Indole	+	+	+
Sodium Pyruvate Creatine	-	-	-
Kohn Charcoal Gelatin	-	-	-
Glucose, Potassium Nitrate	+	+	+
Mantol	-	-	-

Inositol	+	-	+
Rhamnose	+	+	+
Sucrose	-	-	-
Melibiose	-	-	-
Amygdalin	-	-	-
Arabinose	+	-	+
Oxidase	-	-	-
Reduction of Nitrates to Nitrites	+	+	+
Reduction of Nitrates to N <sub>2</sub> gas	-	-	-
Mobility	+	+	+
Oxidation of Glucose	+	+	+
Fermentation of Glucose	+	-	+
Antibiotic Resistance	Km <sup>R</sup> Ap <sup>S</sup> Tc <sup>S</sup>	Km <sup>S</sup> Ap <sup>R</sup> Tc <sup>R</sup>	Km <sup>S</sup> Ap <sup>R</sup> Tc <sup>R</sup>

On the other hand, the individual bacterial strains were analyzed for fatty acid Methyl Esters (FAMES) by gas chromatography.

For the FAMES assay, there was used the Hewlett Packard series II Gas Chromatograph model 5890A (Microbial ID. Inc., Delaware, USA) with a 5 25cm×0.22mm×0.33µm separation column, e.g., a capillary column (HP 19091B-102) fused with methylphenyl silicon.

The gas chromatography was performed under the conditions that the carrier

gas is hydrogen, the column head pressure 10 psi, the split ratio 100:1, the split vent 50 ml/min, the septum purge 5 ml/min, the FID hydrogen flux 30 ml/min, the FID nitrogen flux 30 ml/min, the FID air 400 ml/min, the initial temperature 170 °C, the program rate 5 °C/min, the final temperature 270 °C, the FID temperature 300 °C, the temperature of the inlet port 250 °C, and the injection volume 2 µl.

The FAME graphs were obtained using a microbial identification system software (Microbial ID, Inc., Delaware, USA). A comparison with a standard calibration mixture (Microbial ID. Inc., Delaware, USA) enabled peak identification, and determination of retention time, peak region and peak percentage.

The assay of the FAMES for the respective strains of EBC100, 101 and 103 revealed that the intracellular fatty acids composition is C12:0, C14:0, C16:0, C16:1, C17:0 cyclo and C14:0 3OH for EBC100; C12:0, C14:0, C15:0, C16:0, C17:0 cyclo and C14:0 3OH for EBC101; and C14:0, C15:0, C16:0, C17:0 cyclo, and C14:0 3OH for EBC103.

#### 4. Isolation of Constituent Strains of Bacterial Consortium EBC1000

The following procedures are performed for isolating the individual strains from the bacterial consortium EBC1000.

When cultured in the Luria-Bertani agar solid nutrient medium (containing 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl and 1.5% agar in 950 ml of deionized water), EBC106 (*Bacillus*) is characterized by a thick wrinkle peculiar to the *Bacillus*, EBC107 (Gram negative bacteria) a brownish colony, and EBC108 (Gram positive bacteria) a yellowish colony.

For the other strains the respective colonies can be identified by dilution-smearing the bacterial consortium EBC1000 in desoxycholate agar containing 10 g of

bacto-peptone, 10 g of bacto-lactose, 1 g of sodium desoxycholate, 5 g of sodium chloride, 2 g of dipotassium, 1 g of ferrous citrate, 1 g of sodium citrate, 15 g of bacto agar and 0.03 g of neutral red in 1 liter of deionized water (Difco manual, 1984). After cultured for 24 hours in the desoxycholate agar, EBC100, EBC101 and EBC103 have  
 5 white-in-red spots with viscosity and are slightly different in size, EBC104 white-in-red spots without viscosity, EBC105 brownish spots with viscosity, EBC106 and EBC107 light brownish spots with viscosity, EBC108 colorless spots with viscosity, and EBC109 red spots with viscosity (See. Dictionary of Microbiology and Molecular Biology, 2<sup>nd</sup>, Paul Singleton Diana Sainsbury, 1987).

#### 10 5. Characteristics of Bacterial Consortium EBC1000

(1) Decomposition velocity of sodium alkylaryl naphthalene sulfonate (Tamol-SN) by bacterial Consortium EBC1000

Batches of Tamol-SN with an increasing concentration of 500 ppm, 1000 ppm, 2000 ppm and 4000 ppm were added to the minimal medium (pH 7.2) prepared by  
 15 dissolving 0.065 g of  $K_2HPO_4$ , 0.017 g of  $KH_2PO_4$ , 0.1 g of  $MgSO_4$  and 0.5 g of  $NaNO_3$  in 1 liter of deionized water. After inoculation of the bacterial consortium EBC1000, the absorbance and the Tamol-SN concentration were measured with an elapse of time. The results are presented in Table 3.

[Table 3]

Tamol-SN (ppm)	Decomposition Velocity (mg/l/h)
500	1.3
1000	3.8
2000	5.2
4000	4.0

As shown in Table 3, the decomposition velocity per hour of Tamol-SN was 1.3 mg/l/h at the Tamol-SN concentration of 500 ppm and 4.0 mg/l/h at the Tamol-SN concentration of 4000 ppm.

5 (2) Decomposition velocity of pentachlorophenol compound by bacterial Consortium EBC1000

Batches of pentachlorophenol compound with an increasing concentration of 200 ppm, 500 ppm, 1000 ppm and 2000 ppm were added to the medium prepared by dissolving 20 mg of BTB in 1 liter of deionized water under the same conditions of the  
10 minimal liquid medium as described in (1). After inoculation of the bacterial consortium EBC1000, the absorbance and the pentachlorophenol compound concentration per hour were measured. The results are presented in Table 4.

[Table 4]

Pentachlorophenol Compound (ppm)	Decomposition Velocity (mg/l/h)
200	0.9
500	6.5
1000	5.0
2000	4.5

15 6. Treatment of Recalcitrant Industrial Wastewater with Bacterial Consortium EBC1000

Experimental Example 1: Use of Bacterial Consortium EBC1000 in Treatment of a Recalcitrant Special Emulsifying agent Contained in Petroleum Industrial Wastewater.

1% of crude liquid of sodium alkylaryl naphthalene sulfonate(Tamol-SN), which is the typical recalcitrant chemical used in the petroleum plant, was added to 50 ml of a minimal medium (pH 7.2) prepared by dissolving 0.065 g of  $K_2HPO_4$ , 0.017 g of  $KH_2PO_4$ , 0.1 g of  $MgSO_4$  and 0.5 g of  $NaNO_3$  in 1 liter of deionized water. After inoculating  $2.0 \times 10^4$  /ml of the bacterial consortium EBC1000, shaking culture was performed at 100 rpm, at 25 to 30 °C for 10 days. The results are presented in Table 5.

[Table 5]

Time (day)	Tamol-SN (ppm)	Number of Bacterium (CFU/ml)
0	10000	$2.0 \times 10^4$
2	6000	$3.5 \times 10^5$
3	3600	$3.0 \times 10^6$
4	850	$2.5 \times 10^7$
10	500	$3.0 \times 10^7$

As shown in Table 5, the concentration of Tamol-SN was 850 ppm with the number of bacterium of  $2.5 \times 10^7$  CFU/ml after four days of culture.

Experimental Example 2: Use of Bacterial Consortium EBC1000 in Treatment of recalcitrant crude wastewater from petroleum plants.

Waste acidic and alkaline recalcitrant wastewater to be dumped into the sea was collected from the petroleum plants in the Ulsan industrial complex. The wastewater was crude wastewater contained 4000ppm of sodium alkylaryl naphthalene sulfonate (Tamol-SN), sodium hydrosulfite[ $(NaSO_2)_2$ ], butadiene (BD), acrylonitrile (AN), tetra sodium ethylene diamine tetra acetate dihydrate (EDTA), ferrous sulfate heptahydrate (FES), tert-dodecyl mercaptan (TDDM), paramethane hydroperoxide

(PMH), N-diethyl hydroxy amine (DEHA) and  $\text{CH}_3\text{CN}$  and so on. After inoculating  $1.2 \times 10^7/\text{ml}$  of EBC1000 in 200 ml of the crude wastewater, shaking culture was performed at 80 rpm, at 30 °C for 10 days. The results are presented in Table 6.

[Table 6]

Time (day)	COD <sub>Mn</sub> (ppm)	TOC (ppm)	BOD (ppm)	Number of Bacterium (CFU/ml)
0	4000	3348	610	$1.2 \times 10^7$
4	1882	1670	-	$4.0 \times 10^9$
6	1136	1220	400	$4.8 \times 10^9$
10	750	980	200	$5.1 \times 10^9$

5

As shown in Table 6, the COD value of the crude wastewater was reduced to 750 ppm with the decomposition efficiency of 80% after 10 days of culture. The TOC and BOD values had a downward tendency as the COD value and the number of bacterium was  $5.1 \times 10^9$  CFU/ml with an at least hundred-fold increase.

10 For a reference, the analytical methods used in the experimental examples of the present invention were as follows:

#### 1) COD (Mn) Analysis

The chemical oxygen demand (COD) was measured by the COD Mn method according to the official test method for water pollution and waste treatment (Official No. 1993-42, the Ministry of Environment) and Standard Methods (APHA, AWWA, WPCF, 18<sup>th</sup> ed.). Each 10 ml of crude industrial wastewater or waste materials to be dumped into the sea and the wastewater after decomposition treatment of noxious substances with microorganisms were added to a water bath after addition of  $\text{H}_2\text{SO}_4$



and 0.025N  $\text{KMnO}_4$ , and then heated to 100 °C for 30 minutes. After adding 0.025N  $\text{Na}_2\text{C}_2\text{O}_4$ , the mixture was titrated with  $\text{KMnO}_4$  to measure the actual decrement of the COD.

#### 2) BOD Analysis

5 The biological oxygen demand (BOD) was measured as the amount of dissolved oxygen used for 5 days under the biological reaction at 20 °C according to the official test method for water pollution and waste treatment and Standard Methods.

#### 3) TOC Analysis

10 According to the standard method, the crude waste, wastewater and the time-based samples were centrifuged and the resulting supernatant liquids were analyzed for the total organic carbon (TOC) content with a TOC analyzer (TOC-5000 A, Shimadzu com.).

#### 4) CFU Examination

15 The consortiums of indigenous bacteria and inoculum of the individual treated samples were analyzed by the plate count method according to the standard method for bacteria examination, to measure the growth of the bacterium at the beginning of the test and with an elapse of time by a decrease in the noxious substances.

#### 5) Extraction and Analysis of Chlorine Compound

20 The chlorine compound was extracted and analyzed according to the contents of Korean Patent No. 154295 or Korean Patent Application No. 98-20706, and a paper by the inventor (Journal of Applied Microbiology 85-1-1~8, 1998; Journal of Korea Soil Environment Society, 1-1-39~46, 1996).

Experimental Example 3: Use of Bacterial Consortium EBC1000 in Treatment of Recalcitrant Wastewater from Medicine Manufacturing Plant.

Waste acidic and alkaline recalcitrant wastewater to be dumped into the sea was collected from the medicine manufacturing plants in the Ulsan industrial complex and the middle north region. The waste materials contained medical residues and isopropyl alcohol (IPA), methylene chloride (MC), NaOH, Na<sub>2</sub>SO<sub>4</sub>, dimethylamine (DMA) and methanol and exhibited a high COD<sub>Mn</sub> value of 20,000 to 100,000 ppm. After inoculating  $2.0 \times 10^7$ /ml of EBC1000 in each 200 ml of the wastewater, culture was performed with aeration at 20 to 25 °C for 14 days. The results are presented in Table 7.

[Table 7]

Time (day)	COD <sub>Mn</sub> (ppm)	TOC (ppm)	BOD (ppm)	Number of Bacterium (CFU/ml)
0	38000	34000	34500	$2.0 \times 10^7$
3	12000	21000	-	$3.4 \times 10^7$
5	10800	14600	-	$2.0 \times 10^9$
7	8300	10200	-	$1.4 \times 10^{11}$
10	4000	7200	-	$1.6 \times 10^{10}$
14	3300	4300	4080	$3.6 \times 10^{10}$

10

As shown in Table 7, the COD value of the crude wastewater was reduced to 4000 ppm with the decomposition efficiency of 90% after 10 days of culture. The TOC and BOD values also had a downward tendency as the COD value and the number of bacterium of EBC1000 was  $1.6 \times 10^{10}$  CFU/ml with an at least ten hundred-fold increase.

15

## 7. Tests for Soil and Timber Contaminated by Chlorine Compound

Experimental Example 4: Use of Bacterial Consortium EBC1000 in Treatment of Soil Contaminated by Chlorine Compound.

600 g of the soil contaminated by the pentachlorophenol compound (PCP) was dispersed in 5 liters of tap water stood for one day and added to a slurry apparatus. Compared to decomposition efficiency of the pentachlorophenol compound by the Bu34 strain + 11 decomposition strains (a deposited strain Bu1 presented in Korean Patent Application No. 98-20706 and 10 unidentified strains decomposing pentachlorophenol) + an unidentified indigenous strain (which appeared in a control apart from the decomposition strains and was isolated by a relative comparison in regard to the distribution of the indigenous strain), the novel bacterial consortium EBC1000 showed a slightly higher decomposition efficiency, as seen in Table 8. For example, after 14 days, the decomposition efficiency for removal of the chlorine compound was 96% for the Bu34 strain but 98% for the novel bacterial consortium EBC1000.

[Table 8]

Time (day)	Bu34 + 11 Dec. Strains + Indigenous Strain		EBC1000	
	Absorbance (A660)	PCP (ppm)	Absorbance (A660)	PCP (ppm)
0	0.400	250	0.350	250
2	0.699	113	0.900	100
5	0.710	75	1.180	60
8	0.947	50	1.200	35
10	1.050	38	1.500	20

14	1.164	10	1.560	5
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#### Experimental Example 5

After each 5 g of chip and sawdust of waste timber was added to 65 ml of the minimal liquid medium and, then EBC1000, subjected to shaking culture at 90 to 100 rpm and 30 °C for 90 hours. The results are presented in Table 9. As shown in Table 9, after 80 hours, the decomposition efficiency of pentachlorophenol(PCP) was 98% under inoculation of EBC1000 with a hundred-fold increase in the number of bacterium.

[Table 9]

Time (day)	Chip or Sawdust (Bu1 + Bu34)		Chip or Sawdust (EBC1000)	
	PCP ( $\mu\text{g/g}$ , dry)	Number of Bacterium (CFU/ml)	PCP ( $\mu\text{g/g}$ , dry)	Number of Bacterium (CFU/ml)
0	1080	$1.4 \times 10^7$	1080	$2.5 \times 10^7$
48	-	-	460	$7.9 \times 10^8$
80	-	-	26	$3.0 \times 10^9$
90	27	$5.0 \times 10^8$	20	$3.1 \times 10^9$

10

#### Experimental Example 6

200 g of sawdust of waste timber was added to 250 ml of the minimal liquid medium, followed by adding EBC1000 of  $2.0 \times 10^8/\text{g}$  (timber). Then, moisture and strains were added to the sawdust and the mixture was standed at the room temperature for 200 days (See. "Composting Method", McBain et al., Biodegradation 6, 47-55, 1995). The results are presented in Table 10. As shown in Table 10, the novel bacterial

15

consortium EBC1000 showed higher decomposition efficiency within shorter time than Bu1 or Bu34.

[Table 10]

Time (day)	Bu1		Bu1 + Bu34		EBC1000	
	PCP ( $\mu\text{g/g}$ , dry)	Number of Bacterium (CFU/ml)	PCP ( $\mu\text{g/g}$ , dry)	Number of Bacterium (CFU/ml)	PCP ( $\mu\text{g/g}$ , dry)	Number of Bacterium (CFU/ml)
0	1080	$5.2 \times 10^8$	1080	$1.4 \times 10^{11}$	1080	$2.0 \times 10^8$
10	900	$1.2 \times 10^{10}$	732	$1.4 \times 10^{11}$	700	$7.0 \times 10^{10}$
20	635	$1.9 \times 10^{10}$	655	$1.4 \times 10^{11}$	520	$8.0 \times 10^{10}$
40	408	$1.4 \times 10^{10}$	327	$1.4 \times 10^{11}$	311	$9.0 \times 10^{10}$
60	375	$8.5 \times 10^{10}$	307	$1.4 \times 10^{11}$	230	$9.2 \times 10^{10}$
80	365	$1.6 \times 10^{10}$	299	$1.4 \times 10^{11}$	140	$8.7 \times 10^{10}$
120	330	$8.3 \times 10^9$	311	$1.4 \times 10^{11}$	110	$3.8 \times 10^9$
200	150	$1.0 \times 10^{10}$	145	$1.4 \times 10^{11}$	70	$1.6 \times 10^9$

5 As understood from the above experimental examples, the novel bacterial consortium EBC1000 is useful for efficient decomposition of chlorine compounds as well as a high concentration of recalcitrant toxic chemicals contained in the wastewater to be dumped into the sea.

8. Analytical Test for Decomposition of Recalcitrant Wastewater [COD (Mn  
10 Method)] and Pentachlorophenol Compound by means of the Individual Constituent Strains of EBC1000

The individual pure isolated colonies of EBC100, EBC101, EBC103, EBC104, EBC105, EBC106, EBC107, EBC108 and EBC109 were inoculated into 20 ml of the liquid medium of waste acidic or alkaline wastewater (prepared by diluting 10% waste acid or alkaline wastewater containing 0.65 g of  $K_2HPO_4$ , 0.17 g of  $KH_2PO_4$ , 0.1 g of  $MgSO_4$ , 0.5 g of  $NaNO_3$  and recalcitrant toxic chemicals of medical and petroleum industrial wastewater in 1 liter of deionized water, pH 0 to 14) and then to 20 ml of the liquid medium of chlorine compound (prepared by dissolving 0.65 g of  $K_2HPO_4$ , 0.17 g of  $KH_2PO_4$ , 0.1 g of  $MgSO_4$ , 0.5 g of  $NaNO_3$  and 50 ppm of pentachlorophenol in 1 liter of deionized water, pH 7.2). After more than 10 days of shaking culture at 20 to 30 °C, the COD and the PCP concentration were measured. As a result, the COD and the PCP concentration were decreased by, on average, 85% for EBC100, 67% for EBC101 and EBC103, 50 to 55% for EBC106, EBC107 and EBC108, and about 20% for EBC104, EBC105 and EBC109. This result showed that the individual constituent strains of the bacterial consortium EBC1000 have the ability to decompose the recalcitrant toxic chemicals and chlorine compounds. However, the bacterial consortium EBC1000 showed higher growth and decomposition efficiency than the individual constituent strains.

As described above, the novel bacterial consortium EBC1000 has the ability to rapidly decompose a high concentration of recalcitrant chemicals contained in the industrial wastewater and noxious waste materials to be dumped into the sea and purify soil, timber, seepage water and ground water contaminated by chlorine compounds, thus preventing and recovering excellently environmental pollutions.

Further, the bacterial consortium EBC1000 is useful for treating a high concentration of recalcitrant waste materials to be dumped into the sea, or soil, timber,

seepage water and ground water contaminated by chlorine compounds in a way of aeration, as well as recovering the contaminated timber. Also, the present invention provides a mixture of *Pseudomonas* Bu34 strain (Korean Patent No. 154295) and genus *Klebsiella* Bu1 strain (Korean Patent Application No. 98-20706) used for

5 recovery of contaminated timber or soil.

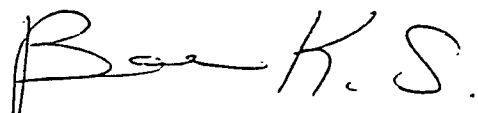
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT  
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

## INTERNATIONAL FORM

## RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: LEE, Sung-Gie  
Keonyeong Apt. 106-304, #2099, Yongam-dong, Sangdang-ku, Cheongju 360-181,  
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:  Mix (EBC1000)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  KCTC. 0652BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [ x ] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on <b>Aug 11 1999</b> .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures  Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):   BAE, Kyung Sook, Director Date: <b>Aug 16 1999</b>



**WHAT IS CLAIMED IS:**

1. A bacterial consortium EBC1000 (KCTC 0652 BP) having an optimum growth under culture in a Luria-Bertani nutrient medium at pH 5 to 8, at a temperature of 25 to 35 °C with shaking at 50 to 100 rpm/min for 24 to 48 hours, the Luria-Bertani nutrient medium containing 10 g of bacto-tryptone, 5 g of bacto-yeast extract and 10 g of NaCl in 1 liter of deionized water, the bacterial consortium EBC1000 being well grown in a successive transfer culture under the same conditions.
2. A method using a bacterial consortium EBC1000 in treatment of waste acidic or alkaline recalcitrant toxic chemicals contained in medical or petroleum industrial wastewater to be dumped into the sea, or chlorine compounds, wherein the recalcitrant toxic chemicals contained in the medical industrial wastewater comprise isopropyl alcohol, methylene chloride, NaOH, Na<sub>2</sub>SO<sub>4</sub>, dimethylamine and methanol, the recalcitrant toxic chemicals contained in the petroleum industrial wastewater comprise sodium hydrosulfite[(NaSO<sub>2</sub>)<sub>2</sub>], butadiene, sodium alkylaryl naphthalene sulfonate, acrylonitrile, tetra sodium ethylene diamine tetra acetate dihydrate, ferrous sulfate heptahydrate, tert-dodecyl mercaptan, para-methane hydroperoxide, N-diethyl hydroxyl amine and CH<sub>3</sub>CN, and the chlorine compounds comprise pentachlorophenol (PCP).
3. The bacterial consortium EBC1000 as claimed in claim 1, comprising EBC100 being a strain of the genus *Klebsiella*.
4. The bacterial consortium EBC1000 as claimed in claim 1, comprising EBC101 being a strain of the genus *Klebsiella*.
5. The bacterial consortium EBC1000 as claimed in claim 1, comprising

EBC103 being a strain of the genus *Klebsiella*.

6. The bacterial consortium EBC1000 as claimed in claim 1, comprising EBC104 being a strain of the genus *Escherichia*.

7. The bacterial consortium EBC1000 as claimed in claim 1, comprising  
5 EBC105 being a strain of the genus *Providencia*.

8. The bacterial consortium EBC1000 as claimed in claim 1, comprising EBC109 being a strain of the genus *Escherichia*.

9. The method as claimed in claim 2, wherein the bacterial consortium EBC1000 comprises EBC100 being a strain of the genus *Klebsiella*.

10 10. The method as claimed in claim 2, wherein the bacterial consortium EBC1000 comprises EBC101 being a strain of the genus *Klebsiella*.

11. The method as claimed in claim 2, wherein the bacterial consortium EBC1000 comprises EBC103 being a strain of the genus *Klebsiella*.

12. The method as claimed in claim 2, wherein the bacterial consortium  
15 EBC1000 comprises EBC104 being a strain of the genus *Escherichia*.

13. The method as claimed in claim 2, wherein the bacterial consortium EBC1000 comprises EBC105 being a strain of the genus *Providencia*.

14. The method as claimed in claim 2, wherein the bacterial consortium EBC1000 comprises EBC109 being a strain of the genus *Escherichia*.

20

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/KR 00/00747

**CLASSIFICATION OF SUBJECT MATTER**

IPC<sup>7</sup>: C12N 1/20; C02F 3/34

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>7</sup>: C12N 1/20; C02F 3/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, CAS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94/29227 A1 (PAQUES B.V.) 22 December 1994 (22.12.94) claims 1,2,11.	1,2
A	US 5705072 A (HAASE) 6 June 1998 (06.06.98) abstract; columns 11, 12; claims 1,2,10. -----	1,2

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents:

„A“ document defining the general state of the art which is not considered to be of particular relevance

„E“ earlier application or patent but published on or after the international filing date

„L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

„O“ document referring to an oral disclosure, use, exhibition or other means

„P“ document published prior to the international filing date but later than the priority date claimed

„T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

„X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

„Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

„&“ document member of the same patent family

Date of the actual completion of the international search

25 October 2000 (25.10.2000)

Date of mailing of the international search report

24 November 2000 (24.11.2000)

Name and mailing address of the ISA/AT

Austrian Patent Office  
Kohlmarkt 8-10; A-1014 Vienna  
Facsimile No. 1/53424/535

Authorized officer

Mosser

Telephone No. 1/53424/437

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.  
PCT/KR 00/00747

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