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ISOLATION OF 2-METHOXYETHANOL DEGRADING BACTERIAL ISOLATE *Pseudomonas sp.* STRAIN VB.

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ABSTRACT

Four bacterial isolates from four different environmental samples (agricultural soil, compost soil and sewage sludge- anaerobic and aerobic wastewaters) were screened for their ability to mineralize 2-methoxyethanol under aerobic conditions. Isolate from anaerobic sewage wastewater was most efficient with an average of 48.6mmol/day mineralisation capacity. Morphological, physiological, biochemical and molecular characterization of the isolate showed that the strain, designated VB is of the genus *Pseudomonas*. 16S rRNA sequence analysis showed that the organism is related to *Pseudomonas putida* at 99.9% and *Pseudomonas plecoglossicida* at 99.8% similarity level. It is related to members of the genus *Pseudomomas* belonging to the rRNA group 1 within the gamma (γ) *Proteobacteria*. The G+C content 64.5±0.8mol% is within the range characteristic of the genus *Pseudomonas*.

KEY WORDS: 2-methoxyethanol, environmental samples, degradation, 16S rRNA and *Pseudomonas* sp. strain VB.

INTRODUCTION

Glycols are compounds composed of one to four of ethylene oxide attached together by ether linkage and terminated with an hydroxyl group. Their extremely high solubility in water accounts for their universal applications (Cox, 1978). The ether linkage is the most common and unifying structural feature which confers to both biological and xenobiotic compounds a high degree of resistance to biological mineralisation (Tidswell *et al.*, 1996). 2-methoxyethanol ($C_3H_8O_2$) is a colourless substance that is highly soluble in water and acetone. It is used in nail polishes and wood staining techniques. It is as well used as a good extracting agent for a mixture of compounds of polychloroethylene, benzene, toluene and xylene – aromatics (Brau-Stromeyer and Meyer, 1995). 2-methoxyethanol is poisonous when ingested orally. It has a lethal concentration of 1500ppm for mice in air (ABC Chemie, 1965; Merck Index, 1989). It impairs fertility and cause harm to the unborn child when pregnant women are exposed to it. Marty and Lock-Caruso (1998) reported that 2-methoxyethanol prolonged the gestation period in rodents due to its inhibition of the gap junctional communication.

Microbial degradation of 2-methoxyethanol under anaerobic conditions has in the past decades received much attention and reports to date show that 2-methoxyethanol can only be mineralized under anaerobic conditions (Tanaka *et al.*, 1986; Tanaka and Pfennig, 1988). This paper investigates the isolation of 2-methoxyethanol degrading bacteria isolate under aerobic conditions.

MATERIALS AND METHODS

Chemical

All the chemicals used were of analytical grade (99.9% pure). The water used was ultrapure double-distilled water. 2-methoxyethanol was obtained from Fluka (Buchs, Switzerland).

Sampling and treatment of environmental samples in relation to the mineralisation of 2-methoxyethanol

Wastewater samples were collected from two disposal basins, the aerobic digester basin (Belüftungensbecken, BB) and the anaerobic digester basin (Vorklarbecken VB), while soil samples were collected from agricultural land (Ackerlandboden, AKB) and compost dump (Kompostboden, KB). The wastewater samples were first centrifuged at 8,000xg at 4°C/10min and the suspensions discarded. The pellets were washed twice with mineral salt medium to remove any endogenous mineral or carbon source. The washed pellets were resuspended in fresh mineral salt medium. To 10ml of mineral salt medium was added 5.0g or 5.0ml of soil and wastewater samples in a 100ml flask. The culture was supplemented with 2-methoxyethanol (126.7mmol) and incubated on a rotatory shaker (200rpm) at 30°C in the dark to discourage the

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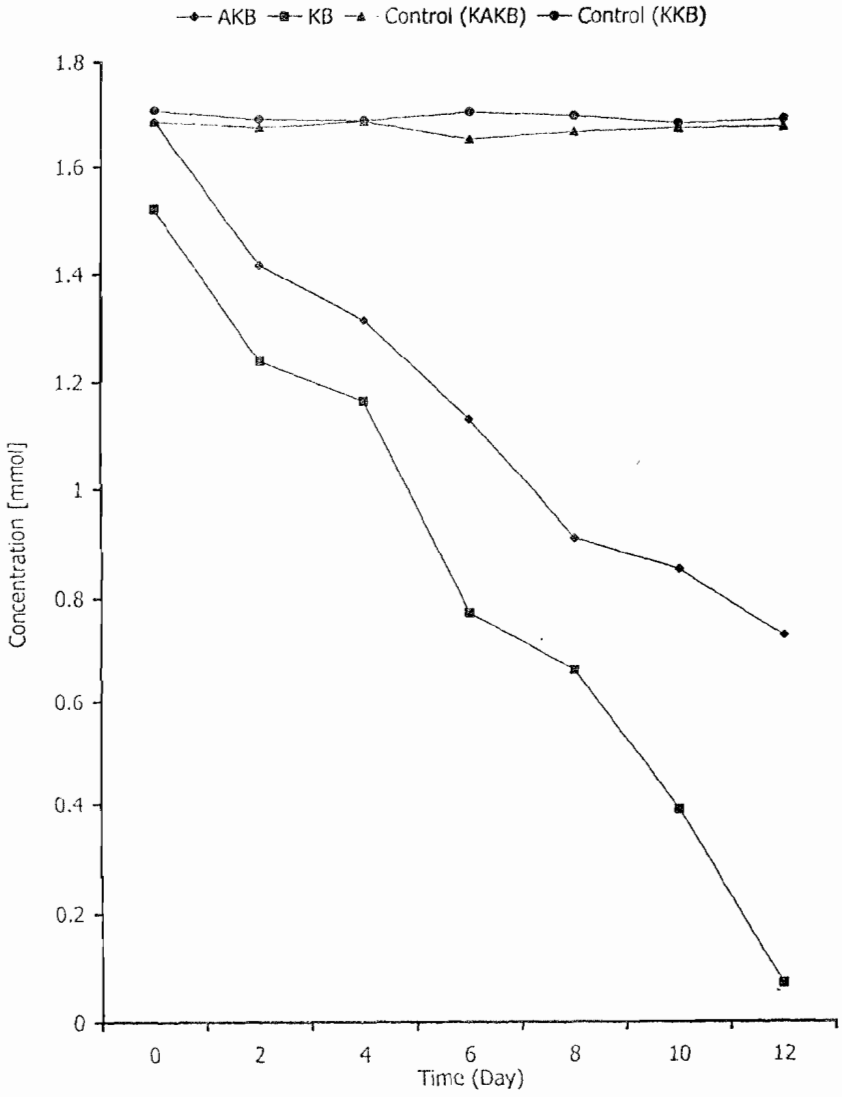

Fig. 1: Microbial mineralization of 2-methoxyethanol in soil samples.

growth of photosynthetic microorganisms. As a control a fifth flask was performed with 1% (w/v) sodium azide (Na_3N). Cultures showing growth were picked for enrichment cultures.

Enrichment and isolation of 2-methoxyethanol utilizing bacteria.

To isolate a bacterial strain able to biodegrade 2-methoxyethanol; the microbial consortium from each of the four environmental samples were used for direct inoculation of 100ml mineral salt medium in 300ml Erlenmeyer flask. The medium was supplemented with 2-methoxyethanol (126.7mmol) as sole source of carbon and energy. Cultures were grown aerobically on a rotatory shaker (200rpm) at 30°C for 24hr. Cultures that showed growth were transferred to fresh medium, and after 3-5 successive transfers, the enrichment cultures were plated onto solidified medium containing 1.5% (w/v) Agar and 0.5% (w/v) each of nutrient broth and yeast extract. Cultures were incubated in incubator pot containing 2-methoxyethanol in the surrounding space. After 2-3days of incubation, the plates were examined for growth. Single colonies were picked and streaked for purity. The pure isolates obtained from the environmental samples were investigated in the liquid medium supplemented with 2-methoxyethanol as sole carbon and energy sources. The isolate with the highest 2-methoxyethanol degrading capacity was picked for further experimentation (Ekhaise, 2002)

Isolation of genomic DNA and determination of DNA base composition

Cells harvested at the exponential growth phase were washed twice with 50mM phosphate buffer (pH 7.2). DNA was isolated and purified by NaOH method (Mesbah *et al.*, 1989). The guanosine - cytosine content (mol % G+C) of the genomi

DNA was determined by HPLC. The nonmethylated Lambda DNA with G+C content 49.858mol% was used as reference.

16S rRNA sequence determination and phylogenetic analysis

The sequence analysis of 16S rRNA of strain VB was determined as described by Rainey *et al.*, (1996). The 16S rRNA sequence manually aligned with reference 16S rRNA from representative microrganism of the γ - subclass of the Proteobacteria, obtained from the EMB Datenbank or Ribosomal Datenbank Project (RDP) (Maidak *et al., 1999).* The evolutionary distance was calculated by the method of Jukes and Cantor (1969). The phylogenetic dendrograms were constructed by using treeing algorithms contained in PHYLIP Package (Felsentein, 1993), and the neighbour – joining method of Saitou and Nei (1987).

Analytical method

The elimination of 2-methoxyethanol in the environmental samples investigated was measured with a gas chromatograph equipped with a flame ionization detector (FID), using a type Packard column Hayesap 80-100mesh, 2m long, inner diameter 2mm (model 430 from Packard, Netherlands) operated isothermally 180°C (oven) and 200°C (injector port and detector).

RESULTS

The environmental samples (AKB, KB, BB and VB) were screened in relation to their elimination capacity of 2-methoxyethanol. The capacity and velocity of 2-methoxyethanol degradation observed with the four environmental samples (Figs. 1 and 2) showed various degree of elimination, with the highest degree of elimination

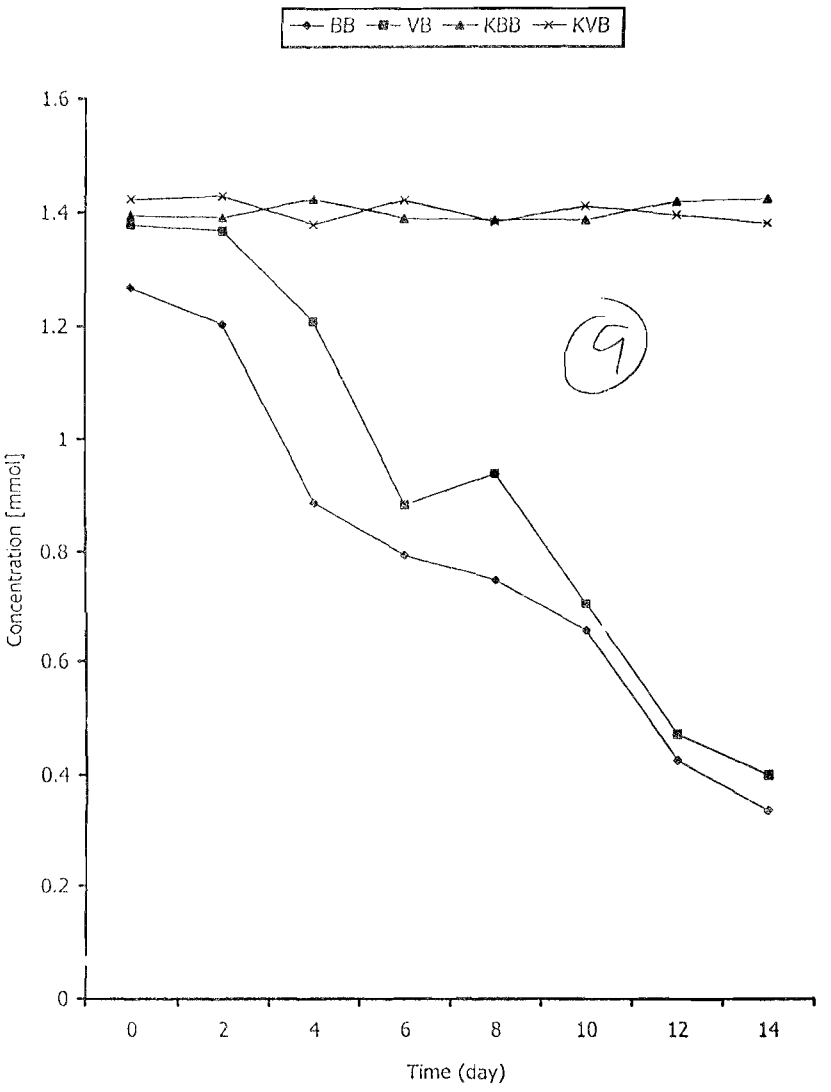

Fig. 2: Microbial mineralization of 2-methoxyethanol in sewage wastewaters.

Table 1: Microbial elimination of 2-methoxyethanol in environmental samples.

Environmental Sample	Elimination activity
Agricultural soil	0.0685 mmol/ml wastewater/day
Compost soil	0.1036 mmol/ml wastewater/day
Aerobic Sewage water	0.0665 mmol/ml wastewater/day
Anaerobic sewage water	0.0699mmol/ml wastewater/day

Table 2: Cultural and Morphological properties of isolate VB

Characteristic	Result
Gram	-
Flagella[1]	Polar lophotrichous
Cell width (μm)[2]	0.968±0.03
Cell length (μm)[2]	2.48±0.7
Colony colour	Brown
Cell form	Rod
Cell capsule	+
Spore formation	-

[1] *Determined by electron microscopy*

[2] *Determined by light microscopy*

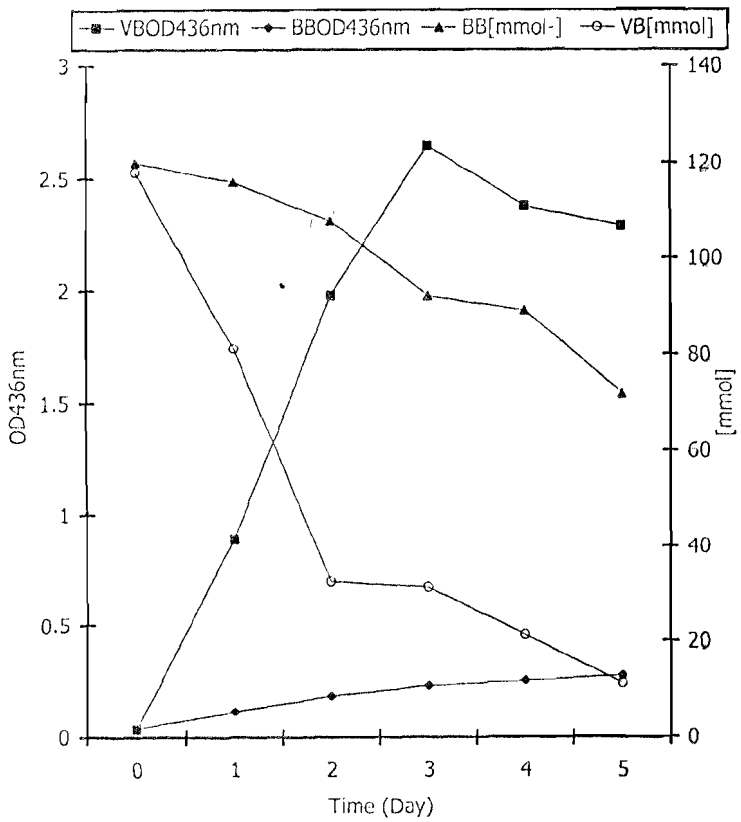

Fig. 3: Microbial elimination of 2-methoxyethanol by bacterial isolates from sewage wastewaters.

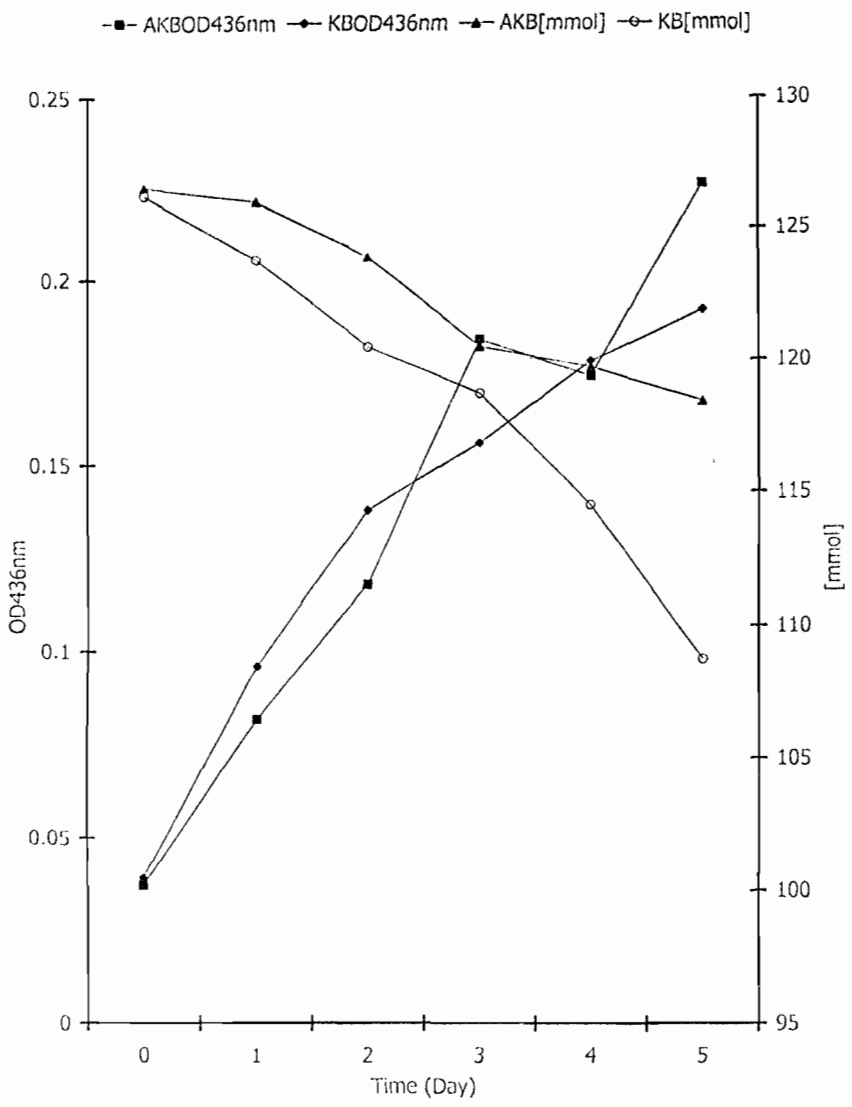

Fig. 4: Microbial elimination of 2-methoxyethanol by bacterial isolates from soil samples.

recorded for soil samples from compost soil. Samples treated with Na_3N showed no loss of 2-methoxyethanol. Sodium azide acts as an inhibitor of protein synthesis and microbial activity, which infers that the elimination of 2-methoxyethanol is a biological process.

Enrichment and isolate capability to mineralize 2-methoxyethanol

Bacterial isolates from each of the environmental samples were screened for the ability to degrade 2-methoxyethanol. The isolate that had the highest degrading ability was picked from each of the samples for further experimentation.

Figs. 3 and 4 showed the microbial activity of the isolated strains. The isolate from the anaerobic sewage sludge was capable of utilizing and degrading 2-methoxyethanol at a faster rate compared to isolate from the aerobic sewage sludge and the soil samples. The utilization capacity of 2-methoxyethanol was most efficient with the isolate from the anaerobic sewage sludge, with an average of 48.6 mmol/day elimination capacity compared to 4.8mol/day, 3.2mmol/day and 2.8mmol/day elimination capacity of the isolates from aerobic sewage sludge, compost soil and agricultural soil respectively. The isolate from the anaerobic sewage sludge designated strain VB, which possess the highest 2-methoxyethanol degrading capacity was selected for further experimentation.

Characteristics of *Pseudomonas* sp. that placed strain VB in the genus *Pseudomonas*.

Strain VB forms small colonies with an entire margin of size 2mm in diameter. It is Gram-negative, non-spore forming straight rod measuring 0.968±0.03µm width and 2.48±0.7µm length (Table 2). It is not pigmented, motile with polar lophotrichous flagella. It possesses oxidase and catalase activity and lacks the urease activity.

16S rRNA sequence analysis

Strain VB is a strict aerobe which possess the chemoorganotrophic mode of nutrient utiiization requiring no growth factor and a G+C content of 64.5±0.8mol%.

The sequence pattern of 16S rRNA of strain VB was compared with those of species of the genus *Pseudomonas*. This was because other morphological, physiological and biochemical characterization indicated strain VB belong to the genus *Pseudomonas*.

DISCUSSION

The critical role of microorganisms in the biodegradation of organic pollutant in the environment has been well investigated (Head, 1998). The focus of glycols research in recent years on the biodegradation of 2-methoxyethanol has resulted in the isolation of a number of microorganisms that can mineralize and utilize 2-methoxyethanol as the sole source of carbon and energy (Tanaka *et al.*, 1986; Tanaka and Pfennig, 1988). These isolates have been used to elucidate metabolic pathways in the mineralisation of 2-methoxyethanol (Tanaka and Pfennig, 1988). However, information on the microbial degradation of 2-methoxyethanol under aerobic conditions is lacking. In this study it was revealed that, the natural microbial flora of certain soil and wastewater samples under aerobic conditions could as well mineralize 2-methoxyethanol known to be biodegraded under aerobic conditions. In this study, the highest elimination activity of 2-methoxyethanol was observed in compost soil (0.1036mmol/g wet soil day). The elimination of 2-methoxyethanol was observed to be due to biological processes since samples treated with sodium azide (Na_3N) showed no loss of 2-methyoxyethanol. The compost soil by its nature is well aerated, and this contains more aerobic microorganisms. This also gave an indication that aerobic microorganisms could also degrade 2- methoxyethanol.

Screening of these various environmental samples indicated that anaerobic sewage harboured the best degrading microorganisms unlike aerobic samples (Fig. 3). The mineralization of 2-methoxyethanol in compost soil of all the soil samples where the highest degree of mineralisation was recorded could be as a result of the effect of cometabolism and synergism, since the best degrading 2 methoxyethanol strain was isolated from the anaerobic sewage wastewater.

Strain VB is a strict aerobe, Gram-negative, nonsporing and rod shaped and possess the chemoorganotrophic mode of nutrient utilization. It is motile with polar lophotrichous flagella. It possess a G+C of 64.5±0.8mol% which falls within the range of 58 – 70 mol% characteristics of the genus *Pseudomonas*. These characteristic features would put strain VB in the genus *Pseudomonas* (Stainer *et al.*, 1984; Palleroni, 1992). It also shares with other members of *Pseudomonas* the ability to utilize a number of carbohydrates as carbon source and possess oxidase and catalase activities.

The phylogenetic analysis of 16S rRNA sequence also shows that strain VB is closely related to members of the genus *Pseudomonas* belong to the rRNA group 1 of the γ-Proteobacteria (Palleroni, 1992). However, strain VB differs from other members of the genus *Pseudomonas* in not possessing fluorescent pigment and its inability to utilize naphthalene, trytophan, anthranilate and acetate, while it utilizes ethylene glycol, glycollate, malate, threonine, nicotinic acid and xylose which other members of the genus *Pseudomonas* cannot utilize.

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