ORIGINAL ARTICLES

Studies on incubation periods, scale up and biodisintegration of poly-β-hydroxybutyrate (PHB) synthesis by *Stenotrophomonas maltophilia* and *Pseudomonas putida*

M. Attia, 1Mohamed T. Shaaban, 2Azza Sh. Turky, 2Nemat M. Awad and 2Eman I. Mowafy

1Botony Dept. Facuty of Sci. Minufiya Univ.
2Agricultural Microbiology Dept. National Research Centre Cairo. Egypt.

ABSTRACT

The present work was carried out to study incubation periods, scale up and biodisintegration of poly-β-hydroxybutyrate synthesis by WN2 (*Stenotrophomonas* sp.) and WN4 (*Pseudomonas* sp.) were found to be the most efficient PHB producers. Poly-β-hydroxybutyrate production by the *Stenotrophomonas maltophilia* and *Pseudomonas putida* strains was investigated in mineral salt medium (MSM) supplemented with the optimum amount of carbon and nitrogen source under the optimum pH value at different incubation times (between 12 h and 138h). The best PHB production and all yields of these strains were determined. Under optimized conditions, *Stenotrophomonas maltophilia* produced a PHB yield of 19.75 g/L after 132h while *Pseudomonas putida* produced 19.16 g/L after 120h, after that, there was a decrease in PHB yields. *Stenotrophomonas maltophilia* performed better than the isolate *Pseudomonas putida* according to their PHB productivity. Scale up studies were conducted in 3L bottle fermentors containing 2L of sterilized MSM broth containing the best C and N sources and maintaining the optimum C:N ratio. Samples were drawn and poly-β-hydroxybutyrate yields determined. *Stenotrophomonas maltophilia* produced a poly-β-hydroxybutyrate yield of 1.954g /100ml culture which is marginally higher than 1.933g/100ml culture produced by *Pseudomonas putida*. The biodegradation of polyhydroxybutyrate (PHB) production by two strains was done by using different soil type. The numbers of PHB degrading microorganisms in natural environments estimated by the clear-zone and film-MPN methods. The numbers of PHB degrading microorganisms differed greatly depending on differences in the natural surroundings. In infertile soil (Wady El-Natron and South El-Tahrir), the numbers ranged between 4.33x10^3/g dry soil and 1.6x10^4/g dry soil, respectively while, in fertile soil (Shalakan Farm), the number was 5.14x10^5/g dry soil.

**Key words:** Poly-β-hydroxybutyrate, *Stenotrophomonas maltophilia* and *Pseudomonas putida*, Different carbon and nitrogen sources, incubation time.

Introduction

Many countries have promoted special programs directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere and have designed novel strategies aimed at facilitating the transformation of contaminants. Biomaterials are natural products that are synthesized and catabolised by different organisms and that have found broad biotechnological applications. They can be assimilated by many species (biodegradable) and do not cause toxic effects in the host (biocompatible) Steinbuchel and Fuchstenbusch, 1998, Zinn, et al., 2001, conferring upon them a considerable advantage with respect to other conventional synthetic products. Bioplastics are a special type of biomaterial. The production of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low polymer production. According to Yamane (1996), the higher production costs, especially raw material costs, make it difficult for poly(3-hydroxyalkanoate (PHA) plastics to compete with conventional petroleum-based plastics in the commercial market place. Hence, alternative strategies for PHA production are being investigated. PHA production costs could be reduced by several means by using cheaper substrates such as starch, whey (Kim *et al.*, 1994) or enhancement of product yield eg., by using recombinant *E. coli* (Lee, 1996).

Approximately 150 different hydroxyalkanoic acids are at present known, as constituents of bacterial storage polyesters (Steinbüchel and Valentin, 1995). PHAs, synthesized by many Gram positive and Gram negative bacteria as storage compounds, are deposited as insoluble inclusions in the cytoplasm (Steinbüchel, 1991). These water insoluble PHAs exhibit rather high molecular weights, thermoplastic and/or elastomeric features and some other interesting physical and material properties. Plastics produced from PHAs have been reported to be truly, fully biodegradable (Page, 1995). Microbes belonging to more than 90 genera including
aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters.

The property that distinguishes PHA from petroleum based plastics is their biodegradability. Biodegradation of PHA under aerobic conditions results in CO₂ and H₂O (Jendrossek et al., 1996), whereas in anaerobic conditions, the degradation products are CO₂ and CH₄. PHA is compostable over a wide range of temperatures, even at a maximum of around 60°C with moisture levels at 55%. Studies have shown that 85% of PHA were degraded in seven weeks (Johnstone, 1990; Flechter, 1993). PHA has been reported to degrade in aquatic environments within 254 days even at temperature not exceeding 6°C (Johnstone, 1990).

In this study, the bacterial strains WN2 and WN4 were identified by 16S rRNA gene analysis, the incubation times, scale up. As soon as, biodisintegration activity of poly-β-hydroxybutyrate (PHB) synthesis by the two stains was done by the formation of a clear zone below and film-MPN methods.

Materials And Methods

**DNA Extraction and PCR Amplification Conditions:**

The genomic DNA of the strain was isolated according to Sambrook et al., (1989). Cells were collected from overnight LB cultures by centrifugation and re-suspended in 500μl TEN buffer. Twenty five μl of 10mg/ml lysozyme were added and the tubes were incubated at 37°C for 30 min, followed by the addition of 75 μl of 10% sodium dodecyl sulphate (SDS). The tubes were inverted gently several times till complete lysis. Then 3 μl of 20 mg/ml of proteinase K were added and the tubes were incubated at 37°C for one hour. After incubation, phenol/chloroform (1:1, v/v) and isoamyl alcohol (24:1) and the tubes were inverted several times and centrifuged for 10 min. The upper phase was transferred to a fresh tube and extracted once with chloroform. The upper phase was again transferred to a fresh tube and 0.7 volume of isopropanol was added and mixed gently and centrifuged for 10 min. The supernatant was removed carefully and the pellets were washed with 1 ml of 70% ethanol. The pellets were collected by centrifugation for 5 min. The DNA was dried and dissolved in 100μl TE buffer and stored at -80°C until use.

Approximately 1500 bp was selected from the polymorphic region of 16S rDNA gene and amplified using specific primers (16S rRNA genes were amplified using universal eubacterial primers 8F (5'-AGAGTTTGATCMTGGCTCAG) and 1492R (5'-GGYTACCTTGTTACGACTT -3'). PCR reaction was performed on the system 9700 thermocycler under the following conditions: 34 cycles of denaturation at 94°C (1 min), annealing at 58°C (1 min) and extension at 72°C (1 min). PCR products were column-purified using QIAquick PCR Purification Kit (Qiagen Valencia, CA, USA). They were sequenced using the universal primer 519F (5'-CAGCAGCCGCGGTAATAC) on an ABI 3730 DNA Analyzer. Gels were photographed by Gel Doc system (Alpha Imager TM1220, Documentation and Analysis system, Canada). Sequencing steps was performed at Gene Analysis unit, VACSERA. Cycle sequencing was done by using a big dye terminator cycle sequencing kit (Applied Biosystems, Foster City). Sequencing products were purified by using Centri-sep spin Column and were resolved on an applied Biosystems Model 310 automated genetic analyzer. Approximately 950 bp was sequenced and phylogenetic and molecular evolutionary analyses of the most efficient poly-β-hydroxybutyrate producing bacterial isolates (WN2 and WN4) based on 16S rDNA genes were conducted using MEGA version 4 (Tamura et al., 2007).

**Estimation of growth curves and PHB production with time:**

The outcome of the orthogonal experiment determined the best incubating conditions of poly-β-hydroxybutyrate production containing the optimum quantities of carbon and nitrogen source and the optimum pH value. 2% (v/v) of the preculture was used to inoculate 250 ml baffled shake flasks containing 100 mL sterile mineral salt medium (MSM) MgSO₄.7H₂O 0.2 g, CaCl₂.2H₂O 10mg, Fe(III)NH₄-citrate 6mg, KH₂PO₄ 0.83 g, Trace elements solution 1ml, Distilled water 1000 ml supplemented with the optimum amount of carbon (glucose) and nitrogen (ammonium sulphate) source under the optimum pH value (7) in a rotary shaker at 30 °C and 200 rpm for 138h. Samples were collected periodically and the growth monitored by measuring the optical density of the culture using Spectrophotometer Model Shimadzu corporation UV-2400PC/2401PC series at a wavelength of 600 nm (Cheesbrough, 2001). Samples were centrifuged at 10,000rpm for 10 min and the pellet washed with acetone and ethanol to remove the unwanted materials. The pellet was resuspended in equal volume of 4 % sodium hypochlorite and incubated at room temperature for 30 min. The whole mixture was again centrifuged and the supernatant discarded. The cell pellet containing poly-β-hydroxybutyrate was again washed with acetone and ethanol. Finally, the polymer granules were dissolved in hot chloroform. The chloroform was filtered. Then the amount of PHB was determined and a curve of PHB production changing with time could be plotted.
Scale up studies:

Based on the performance of 10 promising strains, two potent strains were finally selected for the scale up studies. Scale up studies were conducted in 3 L bottle fermentors containing 2 L of sterilized MSM broth MgSO_4·7H_2O 0.2 g, CaCl_2·2H_2O 10mg, Fe(III)NH_4-citrate 6mg, KH_2PO_4 0.83 g, Trace elements solution 1ml, Distilled water 1000 ml containing the best C and N sources and maintaining the optimum C:N ratio (C=glucose=ammonium sulphate). The medium was bubbled with sterile air by connecting to an air compressor and the strains grown for 144 h. At regular intervals, samples were drawn and PHB yields determined.

The biodisintegration of a bioplastic:

The clear-zone technique used:

Serial dilutions of different soil samples were made in sterile distilled water and the samples were directly poured and plated onto minimal media Na_2HPO_4·2H_2O 9.0g, KH_2PO_4 1.5g, NH_4Cl 0.4g, MgSO_4·7 H_2O 0.2g, CaCl_2·2H_2O 0.02g, Fe(III)NH_4-citrate 1.2mg, Trace elements solution6 0.1ml, Distilled water 1000ml, pH 6.9 (Schlegel et al., 1961) containing 0.03 g of the polymer (poly-β-hydroxybutyrate) as a sole carbon source, which was solidified with 2g agar at pH 7.0 and were incubated at 28 °C for 4 to 5 weeks, The degradation of the polymers by microorganisms were seen as clear zone on the media and the numbers of colonies with clear zones were counted after 5 weeks.

The most probable numbers (MPN technique):

Five ml of the mineral medium was poured into a tube containing a piece of (poly-β-hydroxybutyrate) film with thickness of 0.05 - 0.08 mm (Fig. 3), and the films were stood in the tube using a glass bar, which was sterilized by autoclaving at 110°C for 30 min. The soil samples were diluted at 10^1 ~10^10 with sterile distilled water. One ml of each diluted solution was inoculated into each of 5 tubes. The tubes were incubated at 28°C under aerobic conditions in the dark. The numbers of positive growth tubes were counted after a fixed time. An MPN statistical table was used to determine the growth code and calculate the MPNs (Ishikuri 1992).

Statistical analysis:

The data were analyzed using completely randomized two factorial design (Panse and Sukhatme, 1985). Whenever, the treatment difference is found significant in ‘f’ test, CD was worked out at 1 % probability levels and the values furnished.

Results:

Molecular characterization of the isolates:

The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in GenBank (Fig. 1) revealed that the isolates are similar to Stenotrophomonas maltophilia strain PCP30-HM4394150.1 with 100% similarity and to Pseudomonas putida strain AM921634.1 with 90% as showed in (Table. 1).

According to the phylogenetic tree (Fig.2) based on 16S rDNA sequences, the new two strains were found to be affiliated to the Stenotrophomonas maltophilia (WN2) and Pseudomonas putida (WN4).

Cell Growth Curve and PHB production curve:

The two Strains Stenotrophomonas maltophilia (WN2) and Pseudomonas Putida (WN4) were finally selected based on their performance under optimized conditions. They were investigated for the effect of different incubation periods on their growth (cell count) and PHB productivity. The Table 2 shows the time course of cell growth of the two isolates WN2 and WN4 and its PHB production.

From the Table we could conclude that the PHB production process was a growth-associated process. The PHB was synthesized during cell growth, so the PHB production increased along with cell growth and culture time. But the PHB production began to decrease at 132h in case of WN2 and 138h in case of WN4, we guess that due to the exhaustion of the medium components, intracellularly accumulated PHB could be consumed by microorganism for its maintenance activities. These accords with results published in literature, that is, bacteria synthesize PHB as carbon and energy reserve when nourishment is sufficient and utilize PHB during starvation.
Scale Up Studies Using the Efficient Strains in Optimized Medium:

Scales up studies were conducted in 3 liter bottle fermenters containing 2 liter optimized medium using WN2 and WN4 strains. (poly-β-hydroxybutyrate) accumulation by these strains was monitored every 12 h. PHB yield data are presented in Fig. 3. In all the strains, (poly-β-hydroxybutyrate) started accumulating. Maximum accumulation was found at 96 h and remained stable thereafter. There was no (poly-β-hydroxybutyrate) accumulation during the first 24 hr. Out of two strains tested, the highest (poly-β-hydroxybutyrate) production of 1.10 g/100 ml was obtained by WN2.

The biodisintegration of a bioplastic:

The clear-zone technique used:

The numbers of PHB degrading microorganisms in natural environments estimated by the clear-zone technique (Table 3). The numbers of (poly-β-hydroxybutyrate) degrading microorganisms differed greatly depending on differences in the natural surroundings. In infertile soil (Wady El-Natron and South El-Tahrir), the numbers ranged between 1.6X10^4/g dry soil and 4.33X10^3/g dry soil, respectively while, in fertile soil (Shalakan Farm), the number was 5.14X10^5/g dry soil Figure 4.

The most probable numbers (MPN technique):

The numbers of positive tubes did not change for fertile soil samples after 4 weeks (Table 3). After a week, the solutions in some of the tubes inoculated with different soil samples became opaque. Positive growth was detected by observation of the film pieces in the tube. When each tube was lightly shaken by hand, small pieces of film appeared after 7 days of inoculation with fertility soil and after 15 days of inoculation with infertility soils. After the tubes were further incubated for 3 weeks, a yellowish pigment deposit began to appear on some films near the solution surface in tubes inoculated with the soil samples. Some of the films near the surface of the solution in the positive-growth tubes appeared to be broken after incubating for 4 weeks in the two infertile soils and after incubating for 3 weeks in the fertility soil. The positive-growth tube was the tube in which (poly-β-hydroxybutyrate) film was degraded by (poly-β-hydroxybutyrate) degrading microorganisms. Small pieces of (poly-β-hydroxybutyrate) film appeared and/or the films near the surface of the solution appeared to be broken in the positive-growth tube.

Discussion:

The new two bacterial strains, designated as strain WN2 and WN4, were isolated from newly reclaimed areas (Shaaban, et al., 2011). The bacterial isolate WN2 was found to be affiliated to the genus Stenotrophomonas and was found capable of producing a large amount of (poly-β-hydroxybutyrate), as confirmed by Fourier transform infrared spectroscopy and Ultraviolet spectroscopy analyses (Shaaban, et al., 2011). So far, there hasn’t been any report about bacteria of genus Stenotrophomonas can produce PHB yet. Li et al., (2010) found that the yield of polyhydroxyalkanoates was 23.4% (w/w) based on dried weight of the bacterium cells when HG-B-1 grew in a medium containing saccharose. They analyzed 16S rRNA nucleotide sequence, and ascertained the phylogenetic position of the strain. Strain HG-B-1 with PHAs biosynthesis ability was identified as Stenotrophomonas maltophilia.

S. maltophilia is often found in the rhizosphere and in association with cultivated plants such as maize, potato, wheat, and others (Blondeau, 1980[3]; Garbeva et al., 2001; Heuer and Smalla, 1999; Juhneke et al., 1987; Lambert et al., 1987; Sturz et al., 2001). Moreover, S. maltophilia produces high amounts of indole-3-acetic acid (Berg and Ballin, 1994; Berg et al., 1996). However, this bacterium has been associated with bacteremia, endocarditis, cystic fibrosis, urinary tract infections (Friedman et al., 2002[7]; Khan and Mehta, 2002[16]; Marchac et al., 2004; Vartivarian et al., 1996), and many other diseases in humans (Nicholson et al., 2004; Senol, 2004).

From the figure of time courses of cell growth and PHB production (Fig. 3), we can conclude that the best harvest time in case of WN2 is 132 hr since at that point the (poly-β-hydroxybutyrate) production is highest, (about 19.75g/L) while in WN4 the best harvest time is 120hr since at that point the (poly-β-hydroxybutyrate) production is highest, (about 19.16g/L). Due to the exhaustion of the medium components, intracellularly accumulated (poly-β-hydroxybutyrate) could be consumed by microorganism for its maintenance activities, so the (poly-β-hydroxybutyrate) productions begin to decrease at 138hr in case of WN2 and 132hr in case of WN4, respectively.

Overseas, Lee and Yoo (1994) studied A. eutrophus to produce (poly-β-hydroxybutyrate), the yield was 73.9g/L. Yamane, (1996) investigated Alcaligenes latus to produce (poly-β-hydroxybutyrate) by fermentation
with a production of 68.4g/L. In domestic, the research of (poly-β-hydroxybutyrate) started late than abroad. Du (1997) used \textit{A. eutrophus} and the (poly-β-hydroxybutyrate) production was 49g/L. Yu et al., (2000) from Tsinghua University produced (poly-β-hydroxybutyrate) by engineering bacteria strain VG1 (pTu14) with a (poly-β-hydroxybutyrate) production of 24.6g/L. Researchers of Northwest University used G-y strain to produce PHB, and the yield reached to 21.4g/L. The (poly-β-hydroxybutyrate) productions by other researches were mostly about 0.1mg~15g. Compared with the results published already, the (poly-β-hydroxybutyrate) production of \textit{Stenotrophomonas} is comparatively high. Among natural isolates, \textit{Stenotrophomonas} production is high.

In all strains, (poly-β-hydroxybutyrate) accumulation was increased with time up to 96 h and remained stable thereafter, showing that 96 h was the optimum time of incubation for maximum (poly-β-hydroxybutyrate) synthesis. \textit{Stenotrophomonas maltophilia} WN2 accumulated higher PHB of 1.95 g/100 ml which with \textit{Pseudomonas putida} WN4 1.763 g/100 ml.

Thus the study has come out with an efficient natural (poly-β-hydroxybutyrate) synthesizing bacterial strain \textit{Stenotrophomonas maltophilia} WN2. Which is superior to the \textit{Pseudomonas putida} WN4. Under the optimized conditions, in three liter bottle fermentors. The strain produced 1.95 g/100 ml in 96 h.

The numbers of (poly-β-hydroxybutyrate) degrading microorganisms in natural environments estimated by the clear-zone and MPN method. The numbers of (poly-β-hydroxybutyrate) degrading microorganisms differed greatly depending on differences in the natural surroundings. In infertile soil (Wady El-Natron and South El-Tahrir), the numbers ranged between 4.33x10^3/g dry soil and 1.6x10^5/g dry soil, respectively while, in fertile soil (Shalakan Farm), the number was 5.14x10^5/g dry soil.

The degradation properties of polymer (poly-β-hydroxybutyrate) were studies with the microorganisms from different soil types by using MPN technique. Degradation of (poly-β-hydroxybutyrate) was affected significantly when the PHB containing media was supplemented with consumable carbon sources. Result of supplementation studies have indicated that the degradation of (poly-β-hydroxybutyrate) was significantly retarded by the entire carbon source supplemented in the degrading medium. This is predictable, in view of the biodiversity of the polymer-producing prokaryotes in these environments (Zinn et al., 2001) and the consequent availability of PHB as a nutrient source. Fungi that degrade (poly-β-hydroxybutyrate) and copolymer were mostly isolated from soil compost, garden soil.

Bacteria are considerable degraders of (poly-β-hydroxybutyrate) through their capability of production of intracellular depolymerase enzyme. Hence, their presence in these environmental samples was studied through a basal minimal media containing 0.03% of the polymer as a carbon source. The results indicate that the soil from garden soil exhibited highest number of PHB degrading bacteria. (poly-β-hydroxybutyrate) degrading bacteria secrete specific (poly-β-hydroxybutyrate) depolymerase which hydrolyze the polymer to water soluble monomers or oligomeric esters. The hydrolytic products are taken up by the cells and metabolized.

<table>
<thead>
<tr>
<th>Table 1: Taxonomic relationships of the bacterial isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>6S genes</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

WN2
Microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics (Gu et al., 2000). The biodegradation of bioplastics proceeds actively under different soil conditions according to their properties, because the microorganisms responsible for the degradation differ from each other and have their own optimal growth conditions in the soil. Polymers, especially bioplastics, are potential substrates for heterotrophic microorganisms (Glass and Swift, 1989).

**Fig. 1:** Caliper® 1000 electropherogram for the Two strains WN2 (*Stenotrophomonas maltophilia*) and WN4 (*Pseudomonas putida*).

**Fig. 2:** Phylogenetic tree derived from the 16sRNA sequence of the two isolates WN2 and WN4 and most closely related bacterial species.
Table 2: Cells number and PHB productivity at different culture hours.

<table>
<thead>
<tr>
<th>Number</th>
<th>Time (h)</th>
<th>Cells number (×10^8/mL)</th>
<th>Abs(235nm)</th>
<th>PHB production (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WN2</td>
<td>WN4</td>
<td>WN2</td>
<td>WN4</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>0.24</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.40</td>
<td>0.250</td>
<td>0.121</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>0.80</td>
<td>0.535</td>
<td>0.244</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>1.60</td>
<td>0.808</td>
<td>0.805</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>2.12</td>
<td>0.958</td>
<td>0.956</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>3.30</td>
<td>1.561</td>
<td>1.343</td>
</tr>
<tr>
<td>7</td>
<td>66</td>
<td>3.80</td>
<td>1.523</td>
<td>1.578</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>4.70</td>
<td>1.644</td>
<td>1.724</td>
</tr>
<tr>
<td>9</td>
<td>84</td>
<td>5.78</td>
<td>1.940</td>
<td>1.902</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>6.32</td>
<td>2.245</td>
<td>2.027</td>
</tr>
<tr>
<td>11</td>
<td>96</td>
<td>6.78</td>
<td>2.547</td>
<td>2.394</td>
</tr>
<tr>
<td>12</td>
<td>108</td>
<td>7.11</td>
<td>2.894</td>
<td>2.794</td>
</tr>
<tr>
<td>13</td>
<td>114</td>
<td>7.56</td>
<td>3.093</td>
<td>2.958</td>
</tr>
<tr>
<td>14</td>
<td>120</td>
<td>8.01</td>
<td>3.217</td>
<td>3.189</td>
</tr>
<tr>
<td>15</td>
<td>132</td>
<td>8.37</td>
<td>3.204</td>
<td>3.071</td>
</tr>
<tr>
<td>16</td>
<td>138</td>
<td>8.51</td>
<td>3.097</td>
<td>2.937</td>
</tr>
</tbody>
</table>

* The highest PHB production

Fig. 3: Scale up studies on PHB production (g/100 ml) by the most efficient bacterial isolates under optimized conditions

Table 3: Total microbial counts of PHB degraders in different soil types estimated by clear zone and most probable number.

<table>
<thead>
<tr>
<th>Technique used</th>
<th>Soil Type</th>
<th>infertile soil</th>
<th>fertile soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(WN)</td>
<td>(ST)</td>
<td>(SHF)</td>
</tr>
<tr>
<td>MPN*</td>
<td>1.4x10^4</td>
<td>3.40x10^3</td>
<td>2.90x10^5</td>
</tr>
<tr>
<td>Clear zone</td>
<td>1.6x10^4</td>
<td>4.33x10^3</td>
<td>5.1x10^5</td>
</tr>
</tbody>
</table>

*Average degrader number was calculated from a series of five tubes of tenfold dilution in the MPN statistical

References


