Degradation of polyesters by a novel marine Nocardiopsis aegyptia sp. nov.: Application of Plackett-Burman experimental design for the improvement of PHB depolymerase activity

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This is the first report on the degradation of poly(3-hydroxybutyrate) (PHB), and its copolymers poly(3-hydroxyvalerate) P(3HB-co-10–20% HV) by Nocardiopsis aegyptia, a new species isolated from marine seashore sediments. The strain excreted an extracellular PHB depolymerase and grew efficiently on PHB or its copolymers as the sole carbon sources. The degradation activity was detectable by the formation of a transparent clearing zone around the colony on an agar Petri plate after 25 days, or a clearing depth under the colony in test tubes within 3 weeks. The previous techniques proved that the bacterium was able to assimilate the monomeric components of the shorter alkyl groups of the polymers. Nocardiopsis aegyptia hydrolyzed copolymers 10–20% PHBV more rapidly than the homopolymer PHB. The bacterial degradation of the naturally occurring sheets of poly(3-hydroxybutyrate), and its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was observed by scanning electron microscopy (SEM). The samples were degraded at the surface and proceeded to the inner part of the materials. Clear morphological alterations of the polymers were noticed, indicating the degradative capability of the bacterium. Plackett-Burman statistical experimental design has been employed to optimize culture conditions for maximal enzyme activity. The main factors that had significant positive effects on PHB depolymerase activity of Nocardiopsis aegyptia were sodium gluconate, volume of medium/flask and age of inoculum. On the other hand, MgSO4·7H2O, KH2PO4, K2HPO4 and NH4NO3 exhibited negative effects. Under optimized culture conditions, the highest activity (0.664 U/mg protein) was achieved in a medium predicted to be near optimum containing (in g/L): PHB, 0.5; C6H11O7Na, 7.5; MgSO4·7H2O, 0.35; K2HPO4, 0.35; NH4NO3, 0.5; KH2PO4, 0.35; malt extract, 0.5 and prepared with 50% seawater. The medium was inoculated with 1% (v/v) spore suspension of 7 days old culture. Complete clarity of the medium was achieved after 3 days at 30°C.

Key Words——experimental design; Nocardiopsis aegyptia sp. nov.; PHB depolymerase

Introduction

Poly[(R)-3-hydroxyalkanoic acids] [poly(HAs)] are a class of bacterial storage compounds that are synthesized during unbalanced growth by many Gram-negative and Gram-positive bacteria. Poly(HAs) are deposited intracellularly in the form of inclusions bodies (granules) to levels up to 90% of the cellular dry weight (Jendrossek, 2001; Jendrossek et al., 1996; Steinbüchel and Hein, 2001). In the last two decades more than 100 hydroxyalkanoic acids have been identified as other constituents of PHA; most of them are summarized by Steinbüchel and Valentin (1995). Poly-3-
hydroxybutyrate poly(3HB) is the best-known representative of the PHA family. Microorganisms are able to incorporate up to 60 different monomer types into their storage polymer (Steinbüchel, 1991).

Owing to the thermoplastic properties of the PHAs and their biodegradability, PHAs have attracted industrial interest, and bacteriologically produced PHB and its copolymers with 3-hydroxyvaleric acids have been commercially available in small amounts since 1990. On a fairly large scale, PHA is produced industrially by Zeneca Bio Products (Great Britain) and commercialized as biodegradable plastic under the trade name BIOPOL® (Brandl et al., 1995).

PHA can be biodegraded to water and carbon dioxide or methane by a large variety of ubiquitous microorganisms present in many ecosystems (Budwill et al., 1992; Krupp and Jewell, 1992). The ability to degrade extracellular PHA is widely distributed among bacteria and fungi and depends on the secretion or surface-display of specific PHA depolymerases, which hydrolyze the polymer by surface erosion to water-soluble monomers and/or oligomers (Molitoris et al., 1996; Murphy et al., 1996). Aerobic and anaerobic PHA degrading bacteria were isolated from various ecosystems such as soil, compost, aerobic and anaerobic sewage sludge, fresh and marine water, estuarine sediment, and air (Abou-Zeid et al., 2001; Jendrossek, 2001; Jendrossek et al., 1996).

To our knowledge there are only a few known PHB degraders from marine environments. P(3HB) degrading actinomycetes (Mabrouk and Sabry, 2001), Streptomycetes, have been isolated from soils and compost and they represent nearly a third of the total prokaryotic isolates from those environments (Mergaert et al., 1992, 1993). More recently Klingbeil et al. (1996), Mergaert and Swings (1996) and Manna et al. (1999) have established the versatility of Streptomycetes to degrade P(3HB), P(3HB-co-19% 3HV), P(3HB-co-97% 3HV) and P(3HB-co-70% 3HD).

Species of Nocardiopsis are strictly aerobic, Gram-positive, non acid-fast actinomycetes (Grund and Kroppenstedt, 1990). They are widely distributed and abundant in soil, mildewed grain and clinical material of human and animal origin (Shearer et al., 1983). Little information is available on the versatility of Nocardiopsis species to degrade P(3HB) and copolymers. Therefore the present study aims to evaluate the ability of Nocardiopsis aegyptia, a new species isolated from marine sediments to degrade poly(3-hydroxybutyrate) (PHB), and its copolymers poly(3-hydroxyvalerate) P(3HB-co-10–20% HV). Morphological alterations of the polymer during bacterial hydrolysis were examined by scanning electron microscopy. Optimization of the medium using statistically based experimental design was employed to evaluate the influence of medium components in liquid fermentations on PHB depolymerase production.

Materials and Methods

Microorganism. The organism used throughout this study was locally isolated from marine sediments of the seashore in Alexandria (Ghanem et al., 2000) and identified as a new species of the genus Nocardiopsis. The name Nocardiopsis aegyptia sp. nov., DSM 44442T was proposed and the phylogenetic analysis was carried out (Sabry et al., 2004). The bacterium was maintained on starch nitrate agar slants containing (in g/L seawater): starch, 20; NaNO₃, 1; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.5; FeSO₄·7H₂O, 0.01; agar, 20. pH was adjusted to 7.

Polymeric materials. Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxy-butyrate-co-3-hydroxyvalerate) P(3HB-co-10–20% HV) were obtained as powders from Aldrich (Steinheim, Germany) and ICI (Brillingham, UK), respectively. Circular films (1–1.5 mm thickness) of P(3HB) and copolymers P(3HB-co-10–20% HV) were prepared by solvent casting techniques from chloroform solutions of polyesters. The solution-cast films were aged for at least 1 month to reach equilibrium crystalinity prior to analysis (Bloembergen et al., 1986).

Preparation of polymer suspension. The powdered polymer of PHB (1.0 g) was suspended in 100 ml of seawater; sonicated for 15 min (Ultrasonic Homoge-nizer 4710 series, Cole-Parmer Instrument Co., Chicago, IL, USA) and sterilized separately. The opaque suspension was added aseptically to 900 ml of liquefied sterile agar mineral salt medium, which was then sonicated for another 10 min, agitated, cooled down to 50°C and poured into sterile plates or test tubes (Matavulj and Molitoris, 1992).

The degradation activity. Degradation activity was determined by recording the clearing of the turbid medium containing (in g/L): polymer (as the sole carbon source), 1; KH₂PO₄, 0.7; K₂HPO₄, 0.7; MgSO₄·7H₂O, 0.7; NH₄NO₃, 1; agar, 15. pH was adjusted to 7 (Augusta et al., 1993). The clear zones (di-
ameter in mm) in the two-layer agar plate technique or depth of the clear zone in (mm) in test tubes were measured at daily (first week) or weekly intervals (Manna et al., 1999). Degradation was expressed as the weight loss of polymer with time and calculated according to the equation of Foster et al. (1995).

**Scanning electron microscopy.** Polymer films, which had been incubated in sterile fluid for 2 weeks (sterile incubated controls) or inoculated with bacteria, after selected periods of incubation, were immersed in 4% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2 for primary fixation. Samples were prepared for SEM examination of the surface. Following primary fixation, the material was post fixed in 20 g/L aqueous osmium tetroxide, dehydrated through a graded ethanol series and then dried to the critical point (Molitoris et al., 1996). The dried material was gold coated and examined with a scanning electron microscope Jeol ISM 5300 operating at 15 kV.

**Growth condition and enzyme production.** Basal medium used in optimization contained (in g/L seawater): PHB, 1; C₆H₁₁O₇Na, 5; KH₂PO₄, 0.7; MgSO₄·7H₂O, 0.7; NH₄NO₃, 1; K₂HPO₄, 0.7; malt extract, 1. Aliquots (50 ml) were dispensed in 250 ml Erlenmeyer flasks and inoculated with 2% (v/v) spore suspension prepared by scraping 7 days old culture slant with 10 ml sterile seawater and incubated aerobically on rotary shaker (120 rpm) at 30 °C for 3 days. Cells at the late log phase of growth were harvested by centrifugation at 7,500 × g for 15 min at 4 °C and the clear supernatant was used as the crude enzyme.

**Enzyme assay.** PHB depolymerase activity was assayed at 30 °C by the decrease in turbidity of PHB granules. A stable suspension of purified PHB granules was prepared by using a sonic oscillator (20 kHz, 250 W) for 10 min. The assay mixture (1.0 ml) contained 400 μg of PHB granules, 50 μmol of Tris-HCl buffer (pH 7.5) and 1 μmol of CaCl₂. The reaction was started by addition of the enzyme and followed by the decrease in turbidity of PHB granules, which was measured at 650 nm, using 1 cm light-path cuvettes. One unit of the enzyme was defined as the amount of protein required to decrease the value of A₆₅₀ by 1 per min (Mukai et al., 1993).

**Experimental design.** The Plackett-Burman experimental design, a fractional factorial design (Plackett and Burman, 1946; Yu et al., 1997) was used in this research to reflect the relative importance of various environmental factors on PHB degradation and PHB depolymerase activity in liquid cultures. In this experiment, eleven independent variables were used in twelve combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (−) level was tested. All trials were performed in triplicate and the averages of degradation observation results were treated as the responses. The main effect of each variable was determined with the following equation:

\[ E_x = \frac{(\Sigma M_i + \Sigma M_0)}{N} \]

where \( E_x \) is the concentration effect of the tested variable, \( M_i \) and \( M_0 \) are PHB depolymerase activity in trials where the independent variable \( X_i \) was present in high and low concentrations, respectively, and \( N \) is the number of trials divided by 2. When the sign is positive, the influence of the variable upon PHB degradation is greater at a high concentration, and when negative the influence of the variable is greater at a low concentration. Using Microsoft Excel, statistical \( t \)-values for unequal paired samples were calculated for determination of variable significance. Because three trials of the design could not be measured, the main effects of the variables were calculated taking this fact in consideration.

**Results**

**Degradation of PHB and copolymers**

The degradation pattern of the polymer as detected by measuring clearing depth in test tubes is shown in Fig. 1A. As clearly observed the bacterium was more efficient in degrading 20% PHBV compared to the PHB or 10% PHBV. Figure 1B shows the degradation of PHB in Petri plates as indicated by the large clear zone formed.

When the homopolymer PHB was used, the % of weight loss increased gradually reaching its maximum value (89.94%) after 30 days of incubation (Fig. 2). The presence of a clearing region of the opaque agar medium around the inoculum confirmed the presence of extracellular depolymerase which hydrolyzed the polymers into water-soluble form.

**Scanning electron microscopy of polymers films**

The degradation patterns of three polymers comprising short chain-length hydroxybutyrate (PHB, 10% PHBV, and 20% PHBV) by Nocardiopsis aegyptia.
were examined by scanning electron microscopy. Figure 3 reveals that the surface of uninoculated control sheet of PHB was smooth but possessed well-defined shallow pits (Fig. 3A), while the surface of the PHB polymer exposed to *N. aegyptia* for 25 days had a rough appearance clearly different from that of the control. The surface exhibited numerous irregular erosion pits of variable sizes, which led to an oreolate appearance with only a few patches of less eroded areas. The surface and particularly the pits were covered with growth of *N. aegyptia* (Fig. 3B). In case of the poly(hydroxyvalerate PHBV 10%), the surface of the uninoculated control sheet (Fig. 3C) was smooth and lacked the shallow pits found in PHB. After inoculation and growth of the bacterium for 25 days, the surface was also eroded and comprised numerous irregular pits with none of the original surface remaining (Fig. 3D), demonstrating the high degradation rate of the polymer. For the poly(3-hydroxyvalerate PHV 20%) the surface of the control sheet was smooth (Fig. 3E) whereas the surface exposed to the strain showed a homogenous eroded pattern. The erosion of the polymer was restricted to the surface and sometimes present in the polymer to which numerous bacteria were attached (Fig. 3F).

**Evaluation of environmental factors on PHB degradation using Plackett-Burman experimental design**

The independent variables examined in Plackett-Burman design and their levels are presented in Table 1. The design was applied with different fermentation conditions. The main effect of each variable upon PHB degradation and the production of PHB depolymerase...
enzyme was estimated and presented in Fig. 4. Within the range of the tested levels of variables, the factors that appeared to be of positive effects are sodium gluconate, volume/flask and the inoculum age. On the other hand, high levels of the other variables showed negative effects on PHB depolymerase activity. Statistical analyses of the data (t-test) showed that variations of sodium gluconate, KH$_2$PO$_4$, volume/flask and NH$_4$NO$_3$ in the tested ranges had the most considerable effects on PHB depolymerase activity (Table 1). The interacting effects of KH$_2$PO$_4$ and volume of medium/flask are represented in three-dimensional representations (Fig. 5A). The inhibitory effect of high levels of KH$_2$PO$_4$ on the activity of PHB depolymerase can be partially overcome by simultaneous increase of the volume of medium/flask. Moreover, Fig. 5B suggests that sodium gluconate within the tested ranges and under the present experimental conditions, was

Table 1. Degree of positive or negative effect independent variables on PHB depolymerase produced by *N. aegyptia* according to levels in the Plackett-Burman experimental design.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Symbol</th>
<th>Level -</th>
<th>0 Level</th>
<th>Level +</th>
<th>Main effect</th>
<th>t-value</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB (g/L)</td>
<td>P</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>-0.026</td>
<td>-0.18645 N.S.</td>
<td></td>
</tr>
<tr>
<td>C$<em>6$H$</em>{12}$O$_7$Na (g/L)</td>
<td>G</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>0.187</td>
<td>1.40076 N.S.</td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O (g/L)</td>
<td>Mg</td>
<td>0.35</td>
<td>0.7</td>
<td>1.05</td>
<td>-0.125</td>
<td>-0.31144 N.S.</td>
<td></td>
</tr>
<tr>
<td>K$_2$HPO$_4$ (g/L)</td>
<td>K$_2$</td>
<td>0.35</td>
<td>0.7</td>
<td>1.05</td>
<td>-0.140</td>
<td>-0.2792 N.S.</td>
<td></td>
</tr>
<tr>
<td>NH$_4$NO$_3$ (g/L)</td>
<td>N</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>-0.196</td>
<td>-0.6259 N.S.</td>
<td></td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (g/L)</td>
<td>K</td>
<td>0.35</td>
<td>0.7</td>
<td>1.05</td>
<td>-0.327</td>
<td>-2.77783 S</td>
<td></td>
</tr>
<tr>
<td>Malt extract (g/L)</td>
<td>M</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
<td>-0.070</td>
<td>-0.76185 N.S.</td>
<td></td>
</tr>
<tr>
<td>Inoculum size (%)</td>
<td>S</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>-0.139</td>
<td>-0.37145 N.S.</td>
<td></td>
</tr>
<tr>
<td>Volume/flask (ml)</td>
<td>V</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>-0.229</td>
<td>1.641814 S</td>
<td></td>
</tr>
<tr>
<td>Inoculum age (days)</td>
<td>A</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>0.105</td>
<td>1.313547 N.S.</td>
<td></td>
</tr>
<tr>
<td>Concentration of seawater (%)</td>
<td>C</td>
<td>50</td>
<td>75</td>
<td>100</td>
<td>-0.002</td>
<td>-0.03297 N.S.</td>
<td></td>
</tr>
</tbody>
</table>

Bold type indicates positive effect, italic type indicates negative effect. Significant (S); Non-Significant (N.S).

$^a$t$_{0.1, 0.05}$ = 1.886, 2.920; $^b$t$_{0.1, 0.05}$ = 1.476, 2.015; $^c$t$_{0.1, 0.05}$ = 1.440, 1.943; $^d$t$_{0.1, 0.05}$ = 1.415, 1.895.

Fig. 4. Elucidation of fermentation factors affecting PHB depolymerase activity produced by *N. aegyptia*.

Fig. 5. The interaction of sodium gluconate with volume/flask (A) and KH$_2$PO$_4$ with volume/flask (B) with respect to PHB depolymerase activity based on the Plackett-Burman experimental results.
very much less effective when compared with the volume of medium/flask. According to the data obtained from the Plackett-Burman experimental results, KH$_2$PO$_4$, K$_2$HPO$_4$ and NH$_4$NO$_3$ should be added in the lowest concentration and the medium predicted to be near optimum should be of the following composition (g/L): PHB, 0.5; C$_6$H$_{11}$O$_7$Na, 7.5; MgSO$_4$·7H$_2$O, 0.35; K$_2$HPO$_4$, 0.35; NH$_4$NO$_3$, 0.5; KH$_2$PO$_4$, 0.35; malt extract, 0.5. Cultures should be inoculated with 1% (v/v) inoculum of 7-day-old culture in a total culture volume of 100 ml 50% seawater. Under pre-optimized culture conditions, complete clearance of PHB-containing culture was achieved after 3 days.

Discussion

This study provides novel information on the degradation of poly-hydroxybutyrate (PHB) by a new species of *Nocardiopsis*. *N. aegyptia* sp nov., which is a halophilic actinomycete isolated from marine sediment. The bacterium grew well on PHB and its copolymers poly(3-hydroxyvaleric acids) poly(3HV) as sole carbon sources. Its degradative efficiency was observed as clear depths in turbid test tubes. This method proved to be more advantageous for assaying degradation activity in fungi and Streptomycetes (Mabrouk and Sabry, 2001; Matavulj and Molitoris, 1992). Degradation of PHB was also followed in Petri plate and the clear zone formed proved the hydrolysis of the polymer. These results are in accordance with those previously published (Augusta et al., 1993; Doi et al., 1992) which suggested that the polymer hydrolysis depends on excretion of exoenzymes, diffusion through the surrounding medium and interaction between the enzymes and the polymer.

The degradation of the three polymeric films was observed using SEM. Polymer sheets incubated in sterile medium had no detectable morphological effect on the exposed polymer surface. In contrast to controls, the surfaces of polymers that had been incubated with the bacterium exhibited numerous irregular erosion pits. It is assumed that the mycelial growth of the bacterium penetrates the lesions, depolymerases are secreted and the hydrolysis products increase. The same observation has been recently reported for *Streptomyces* sp. SNG9 (Mabrouk and Sabry, 2001). The erosion patterns of the different polymers differed and the surface of degraded PHB, 10% PHBV and 20% PHBV polymers had the most homogenous pattern. This might be due to the difference in heterogeneity of the polymer, with its different components exhibiting different susceptibility to enzymatic hydrolyses.

In liquid culture with PHB as the sole carbon source, complete clarity of the turbid medium was achieved after 7 days of incubation with *N. aegyptia*. In spite of this fact, low activity was detected in the medium, indicating that hydrolysis of PHB particles requires direct contact with bacterial cells which might be attributed to the existence of enzyme surrounding the membrane with low amounts released extracellularly.

In order to optimize enzyme production, the Plackett-Burman design was applied, which has been demonstrated as an efficient approach to screen for medium components and/or factors affecting PHB degradation. Increasing gluconate level, volume of medium/flask and age of inoculum proved to enhance polymer hydrolysis. On the contrary, increasing PHB level negatively affected enzyme activity. This led to the suggestion that the *N. aegyptia* PHB depolymerase is constitutive. Our results are in contrast to all other known PHB bacterial depolymerases, which are repressed in the presence of a soluble carbon source (Jendrossek et al., 1995). In accordance with our results are those reported for *Pseudomonas lemoignei* (Nakayama et al., 1985; Steinbüchel and Hein, 2001; Tomasi et al., 1996), which produced polymerase maximally during growth on succinate. This result is in good agreement with a previous investigation which demonstrated the importance of carbon sources in the growth medium for enzyme production as the rate of polymer degradation was influenced by the degree and availability of secondary carbon and by the initial carbon in the liquid medium (Foster et al., 1995). Raising the medium volume/flask exerted a significant positive effect on enzyme activity, which might be explained by the fact that the bacterium requires low aeration. The importance of using inoculum from an old culture coincides with the fact that many of the extracellular degradative enzymes are optimally expressed post-experimentally (Ferrari et al., 1993). Increasing the levels of other medium components had a negative effect on enzyme production. This result might be due to the malt extract incorporated in the medium.

The levels of examined independent variables were calculated and applied in a verification experiment. The great similarity between the predicted and the observed results (0.664 U/ml) proves the accuracy of the model and its application validity. Under optimized
condition, complete hydrolysis of PHB was achieved after 3 days. This value is similar to that obtained for the marine bacterium *Marinobacter* sp. NK-1 when cultivated on P(3HB-3-HB). A relatively similar result was achieved with *Comamonas testosteroni* when grown on P(3HB-co-36% HV); it was found that *C. testosteroni* induces secretion of the enzyme in the presence of water-soluble oligomers of 3-hydroxybutyric acid (Kasuya et al., 1994). On the other hand the optimum activity of *Pseudomonas stutzeri* YM1414 depolymerase when P(3HB-3-HB) used was slightly higher than that obtained by *N. aegyptia* (Mukai et al., 1994). On the other hand, the PHB depolymerase activity found in the halophilic of *N. aegyptia* sp. nov., was about twice that obtained from a strain of *Alcaligenes faecalis* (Tanio et al., 1982).

**References**


Plackett, R. L. and Burman, J. P. (1946) The design of optimum Fig. 1B. *Biometrika*, **33**, 305–325.


