Biodegradation of Glyceryl Trinitrate by *Penicillium corylophilum* Dierckx

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Penicillium corylophilum* Dierckx, isolated from a contaminated water wet, double-base propellant, was able to completely degrade glyceryl trinitrate (GTN) in a buffered medium (pH 7.0) containing glucose and ammonium nitrate. In the presence of 12 mg of initial fungal inoculum, GTN (48.5 to 61.6 μmol) was quantitatively transformed in a stepwise process to glyceryl dinitrate (GDN) and glyceryl mononitrate (GMN) within 48 h followed by a decrease in the GDN content with a concomitant increase in the GMN level. GDN was totally transformed to GMN within 168 h, and the complete degradation of GMN was achieved within 336 h. The presence of glucose and ammonium nitrate in the growth medium was essential for completion of the degradation of GTN and its metabolites. Complete degradation of GTN by a fungal culture has not been previously reported in the literature.

Glyceryl trinitrate (GTN) is one of the major ingredients in double-base propellant compositions (17), which are used in gun and rocket formulations and for the casing of intercontinental ballistic missile motors. The manufacture of GTN, the generation of scrap propellant, and the need to demilitarize the munitions produce large amounts of waste materials containing GTN. Demilitarization of these items has become a significant environmental concern in recent years. Current disposal techniques such as open-air burning and incineration, which produce hazardous waste, coupled with the mammalian toxicity and genotoxicity of GTN (5, 9, 17), point out the need for more environmentally friendly disposal methods. Although several chemical methods for the disposal of GTN have been reported, they are not desirable because of incomplete degradation, large consumption of chemicals, evolution of toxic or offensive gases, and their relatively high cost (1). However, microbial degradation processes hold the potential for more environmentally acceptable alternatives to the current disposal methods (open-air burning, open-air detonation, and incineration).

The aerobic and anaerobic microbial degradation of GTN has been previously investigated (2–4, 11, 19, 22). None of these investigations reported the complete denitrification of GTN. Ducrocq et al. (3, 4) showed that certain yeast and mycelial fungi can convert GTN to a mixture of glyceryl dinitrates (GDNs) and glyceryl mononitrates (GMNs). Wendt et al. (19), in their pioneering studies, proposed that the bacterial metabolism of GTN occurs via sequential denitrification to GDN and GMN and speculated that the GMN was further denitrated to form glycerol. However, the degradation was not complete, as GDN and GMN were still present in the spent medium (19). Recently, Meng et al. (10) reported that GTN can be completely denitrated during a long-term incubation with cell extracts of either *Bacillus thuringiensis* plus *Bacillus cereus* or *Enterobacter agglomerans*. Although the method of Meng et al. appears to effectively degrade GTN, it must be pointed out that *B. cereus* and *E. agglomerans* are mammalian pathogens whereas *B. thuringiensis* is an insect pathogen (6, 12, 16). Further, the authors pointed out that this method is suitable only for the small-scale degradation of GTN (10).

Previously, we reported the isolation of a fungal culture, which was identified as *Penicillium corylophilum* Dierckx, from a water wet, contaminated double-base propellant (14). Of all the ingredients in double-base propellants, only GTN has an appreciable aqueous solubility. It is likely that the organism was utilizing GTN for growth. We previously demonstrated its ability to degrade nitrocellulose (14). To use this culture for the disposal of double-base propellant formulations, it was necessary to study its ability to degrade other major ingredients present in the composition. Hence, we carried out a systematic study of the biodegradation of GTN by *P. corylophilum*.
TABLE 1. Time course of GTN degradation by P. corylophilum Dierckx

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>GTN (μmol)</th>
<th>GDN (μmol)</th>
<th>GMN (μmol)</th>
<th>Total (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48.5</td>
<td>0</td>
<td>0</td>
<td>48.5</td>
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<td>19</td>
<td>37.5</td>
<td>3.0</td>
<td>1.5</td>
<td>42.0</td>
</tr>
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<td>48</td>
<td>0</td>
<td>16.0</td>
<td>10.0</td>
<td>26.5</td>
</tr>
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<td>92</td>
<td>0</td>
<td>3.0</td>
<td>5.5</td>
<td>8.5</td>
</tr>
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<td>164</td>
<td>0</td>
<td>0</td>
<td>18.0</td>
<td>18.0</td>
</tr>
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<td>260</td>
<td>0</td>
<td>0</td>
<td>7.0</td>
<td>7.0</td>
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<td>284</td>
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<td>0</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>308</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

* Initial GTN content, 48.5 μmol. The experiment was conducted in a single flask, and 15 μl was used at each time point for HPLC analysis.
* The mobile phase containing 60% acetonitrile and 40% water was used for the HPLC separation of GTN and its products.
* The mobile phase containing 5% acetonitrile and 95% water was used.

RESULTS AND DISCUSSION

The initial experiment directed towards the degradation of GTN by P. corylophilum (biomass dry weight, 4.0 mg) with 0.6% glucose but without an additional nitrogen source resulted in 51% of the GTN (initial content, 11 μmol) being transformed to a mixture of GDN and GMN by 166 h with an increase in the biomass weight to 19.3 mg. This result is consistent with those of Duroc et al. (3, 4), who showed that Phanerochaete chrysosporium and Geotrichum candidum can convert GTN to a mixture of GDN and GMN in the presence of a carbon cosubstrate. Attempts were made to improve the degradation by adding 0.08% ammonium nitrate as a supplemental nitrogen source to the growth medium (18). By 166 h, about 64% of the GTN was transformed to a mixture of GDN and GMN.

Efforts were made to identify the culture conditions required to achieve complete degradation of GTN by P. corylophilum (biomass dry weight, 4.0 mg) with 0.6% glucose but without additional nitrogen sources. Based on a series of preliminary studies, it was found that the inoculum size, amounts of additional carbon and nitrogen sources, and pH were major factors that affected the extent and rate of degradation. The preliminary experiments were carried out at 0, 0.1, 0.2, 0.3, 0.4, and 0.5% ammonium nitrate along with glucose as a supplemental carbon source. In the absence of ammonium nitrate, the GTN was converted to a mixture of GDN and GMN. Upon addition of lower levels of ammonium nitrate, the GDN was converted to GMN. It appears that the amount of nitrogen available from GMN was not sufficient to support the fungal growth. Complete disappearance of GTN (48.5 to 61.6 μmol) was obtained with 12 mg (dry weight) of P. corylophilum in 50 ml of growth medium containing 8% glucose and 0.4% ammonium nitrate buffered at pH 7.0 with 130 mM phosphate buffer.

A systematic time course study was then conducted with the reagents in a single flask. In this study, it was observed that the GTN was absent from the medium after 48 h and only GDN (1.2-GDN and 1.3-GDN) and GMN (1-GMN and 2-GMN) remained (Table 1). The GTN content decreased with time and was zero at 164 h with only GMN remaining in the medium. The degradation of GMN was much slower and was completed by 308 h. A significant loss in the mass balance of GTN was observed in the medium before the formation of GMN. This is not consistent with the stepwise degradation mechanism proposed by earlier investigators (8, 13, 15, 19, 20).

Because of the apparent lack of mass balance and the presence of a large quantity of biomass in the growth medium (Table 2), we studied the possibility of the adsorption of GTN and its degradation products by the biomass. This was done in another set of experiments in individual flasks by filtering off the biomass from the medium and thoroughly washing it with water. The biomass was then homogenized and centrifuged, and the pellet was extracted with methanol and centrifuged (8,000 x g) at 28°C for 15 min. The biomass was homogenized (8,000 x g) at 28°C for 15 min.

The GTN, GDN, and GMN contents in the washings, aqueous homogenate supernatant, and methanol were determined (Table 3). A significant amount of GTN and its degradation products were detected in the washings and in the medium. However, no GTN or its degradation products were detected in the aqueous homogenate supernatant or in the methanol extract of the pellet.

A gradual decrease in the amount of adsorbed material was observed up to the 72-h time point (Table 3). At 168 h, no adsorbed GTN or degradation products were detected. By adding up the number of micromoles of GTN and its degra-
modification of this technology.

select a less expensive nitrogen source for the practical imple-
support fungal growth and thus to produce the enzyme system
necessary for the production of the enzymatic system respon-
GMN. It appears that the presence of ammonium nitrate is
in the culture medium. The data indicates that at least 0.4% ammo-
GDN and GMN. In the presence of low levels of ammonium
been previously reported. In the present investigation, a sig-
pathogenic fungal culture,
P. corylophilum
studies including identification, purification, and characteriza-
tion of the enzymes involved in the GTN degradation are
control (HgCl2 and NaN3).

Overall, GTN can be completely degraded by using a non-
pathogenic fungal culture, P. corylophilum. To the best of our
knowledge, the complete fungal denitrification of GTN has not
been previously reported. In the present investigation, a sig-
ificant increase in the biomass content was observed during
the GTN denitration process (Table 2). The biomass data
indicates that the GTN, GDN, and GMN removal occurred
during fungal growth. Our results show that in the absence
of ammonium nitrate, the GTN was converted to a mixture of
GDN and GMN. In the presence of low levels of ammonium
nitrate, significant amounts of GMN were still present in the
culture medium. The data indicates that at least 0.4% ammo-
nium nitrate is necessary for the complete denitrification of
GMN. It appears that the presence of ammonium nitrate is
necessary for the production of the enzymatic system respon-
sible for the denitrification of GMN. It is likely that the nitrogen
released from the GDN-to-GMN conversion was insufficient to
support fungal growth and thus to produce the enzyme system
required for GMN denitrification. Once the enzymatic mecha-
nism for GMN degradation is elucidated, it may be possible to
select a less expensive nitrogen source for the practical imple-
mentation of this technology.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Initial amt (μmol) of GTN</th>
<th>Amt (μmol), in growth medium, of:</th>
<th>Total amt (μmol)</th>
<th>Amt (μmol) of GTN adsorbed to biomass</th>
<th>Total amt (μmol) of GTN and degradation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(^a)</td>
<td>59.1</td>
<td>59.1</td>
<td>0</td>
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<tr>
<td>19(^b)</td>
<td>58.0</td>
<td>39.7</td>
<td>7.6</td>
<td>0</td>
<td>0</td>
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<tr>
<td>24(^b)</td>
<td>58.9</td>
<td>34.0</td>
<td>7.9</td>
<td>4.7</td>
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<td>48(^b)</td>
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<td>0</td>
<td>14.6</td>
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<td>1.5</td>
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<td>72(^b)</td>
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<td>4.1</td>
<td>3.4</td>
<td>9.1</td>
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<td>168(^b)</td>
<td>61.6</td>
<td>0</td>
<td>0</td>
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<tr>
<td>336(^b)</td>
<td>60.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16.6</td>
</tr>
</tbody>
</table>

\(^a\) The mobile phase containing 60% acetonitrile and 40% water was used for the HPLC separation of GTN and its products.

\(^b\) GTN and its degradation products remaining in the growth medium and adsorbed to the biomass. About 99.5% of the added GTN was accounted for in the killed control (HgCl\(_2\) and NaN\(_3\)).

REFERENCES


