The Analysis of *Rhodococcus ruber* in Different Growth Conditions Using Pyrolysis Mass Spectrometry (PyMS)

Ergin KARIPTAŞ1,2, Kamil İŞIK1,2, Nevzat ŞAHİN1,2, Reşit ÖZKANCA1, Alan C. WARD2*

1Department of Biology, The Faculty of Science and Letters, Ondokuz Mayıs University, 55139, Kurupelit / Samsun - TURKEY

2Department of Microbiology, The Medical School, University of Newcastle upon Tyne, NE2 4HH, ENGLAND

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**Abstract:** Batch grown cultures of *Rhodococcus ruber* from different growth phases and continuous grown cultures from carbon, phosphate and magnesium limitation were analysed by pyrolysis mass spectrometry (PyMS). Batch grown cells showed significantly different pyrograms and the stationary phase cells could be clearly distinguished from exponential phase cells. Mass pyrograms of carbon, phosphate and magnesium limited cells were clearly different and, for carbon and phosphate limitation, clear trends were seen with different dilution rates. Changes in pH and temperature had no systematic effect on mass pyrograms.

**Key Words:** *Rhodococcus ruber*, PyMS, Environmental conditions, Continuous culture

Farklı Ortam Şartlarında Yetiştilirilen *Rhodococcus ruber*’in Pirolizis Kütle Spektrometri (PyMS) ile Analizi

**Özet:** Kapalı kültür ortamında (kesikli sistem), farklı büyüme evrelerinde yetişen, *Rhodococcus ruber*’in saf hücre örnekleri ile açık kültür ortamında (kesikli sistem), karbon, fosfat ve magnezyum limitlenmesi yapılarak yetişen hücre örnekleri, pirolizis kütle spektrometrisi (PyMS) ile analiz edilmisti. Kapalı kültür ortamında yetişen hücreler farklı piyogramlar göstermiş ve durgun fazdaki hücrelerin, logaritmik fazdaki hücrelerden açık bir şekilde ayrıldığı belirlenmiştir. Karbon, fosfat ve magnezyum limitlenmesi yapılan hücrelerin kütle piyogramları belirgin bir şekilde farklıdır ve farklı oranlardaki besi yeri ağız hıza bağlı olarak karbon ve fosfat limitlenmesi yapılan piyogramlarda açık eğimler görülmektedir. Sıcaklık ve pH değişimlerinin kütle piyogramları üzerine sistematik bir etkisi olmamıştır.

**Anahtar Sözcükler:** *Rhodococcus ruber*, PyMS, Çevresel koşullar, Sürekli kültür

* Present address: Department of Agricultural and Environmental Science, Newcastle University, Newcastle upon Tyne NE1 7RU, UK
Introduction

The actinomycete genus *Rhodococcus* (1-3) is a taxon which produces enzymes that mediate xenobiotic transformations (4) and degrade diverse pollutants (5). Rhodococci may have a role to play in bioremediation and detoxification of contaminated soils and may prove to be valuable sources of antibiotics (6) and biosurfactants (7). Recent information indicates that rhodococci of animal origin are opportunistic human pathogens, showing the need for greatly improved recognition and understanding of the virulence factors associated with the genus *Rhodococcus* (2, 3, 8). Despite their importance in natural habitats and microbial biotechnology, relatively little is known about the biology of rhodococci.

Curie-point pyrolysis (PyMS) mass spectrometry has been increasingly applied in microbial systematics, in the classification, identification, comparison, characterisation and discrimination of microbial strains. In these applications, the organisms are grown under standard conditions and the variation of chemical composition is a reflection of the genotype of the organism (9-16).

PyMS has been successfully applied to monitor the inter-strain comparison and changing chemical composition of micro-organisms with growth and environmental conditions (17-19). In recent years, similar studies have been performed and it has been reported that PyMS is a very powerful and useful tool for analysing environmental effects on the chemical composition of bacteria (18, 20-22).

Materials and Methods

**Growth conditions:** *Rhodococcus ruber* JCM 0205 (JCM, Japanese collection of microorganisms), shaking at 160 rpm at 30°C, was grown in batch cultures in the mineral salts medium (23) supplemented with 0.028 g thiamine and 15 g glucose at the following concentrations: Na₂HPO₄·2H₂O, 3.57 g; KH₂PO₄, 1.5 g; NH₄Cl, 1.0 g; MgSO₄·H₂O, 0.2 g; CaCO₃, 0.02 g; FeSO₄·7H₂O, 0.01 g; H₃BO₃, 50 µg; CuSO₄·5H₂O, 10 µg; MnSO₄·4H₂O, 40 µg; ZnSO₄·7H₂O, 40 µg; Na₂MoO₄·2H₂O, 24 µg; distilled water, 1 litre; pH 7.2. The cultures grown for 20 hours correspond to the early exponential phase, the cultures grown for 44 hours correspond to the late exponential phase, and the ones grown for 96 hours correspond to the stationary phase.

Continuous cultivation was carried out in a 2 litre fermenter system (LSL Ltd., UK). The mineral salts medium (23) was used to obtain carbon, phosphate and magnesium limitations. In the carbon-limited culture, the concentration of glucose was decreased to 6.4 g/l. For phosphate limitation, the mineral salts medium contained (per litre): Na₂HPO₄·2H₂O, 0.714 g; KH₂PO₄, 0.3 g; K₂SO₄, 1.93 g; and NH₄Cl, 1.0 g. In magnesium-limited culture the concentration of MgSO₄·H₂O, was 0.0125 g in 1 litre.
Carbon- and phosphate-limited-continuous cultures were established over a range of dilution rates (0.028 to 0.121 h⁻¹), temperatures (25° to 37°C) and pH levels (5.93 to 7.93). A single magnesium-limited culture was established at a dilution rate of 0.028 h⁻¹, a temperature of 30°C and pH of 6.91; growth could not be established under other conditions tried.

The culture samples were harvested from chemostat and batch cultures. The samples were centrifuged and washed twice with phosphate buffer and finally with distilled water. They were frozen, freeze dried and then stored at -20°C (24).

Sample preparation: Pyrolysis foils and tubes (Horizon Instruments Ltd.) were put in acetone and dried overnight. Flamed forceps or sterile plastic loops were used to insert each foil into pyrolysis tubes in such a way that they protruded about 6 mm from the mouth. The freeze dried samples were resuspended in 100 µl of distilled water and samples (ca. 50 mg wet weight) were uniformly streaked onto Fe-Ni (ferro-nickel) foils. The pyrolysis foils were allowed to dry at room temperature for an hour and then dried at 80°C for 10 minutes. For analysis, the foils were tamped into tubes with a flamed stainless steel tool (gagua) so that they lay 10 mm from the mouth. Viton 'O'-rings were placed on the tubes, which were then loaded onto the pyrolysis mass spectrometry carousel in batches of 150. Samples were prepared in triplicate.

Pyrolysis mass spectrometry: Pyrolysis mass spectrometry was carried out using a Horizon Instruments RAPyD 400X pyrolysis mass spectrometer (Fig. 1). The inlet heater control was set to 140°C, and the heated tube loader to 80°C. The assembled tubes were loaded in sequence into the pyrolysis chamber by a robotic arm. An oscillating RF frequency current was applied to the pyrolysis coil and heated the pyrolysis foils rapidly, with a 0.6 second
rise time, to the Curie point of 530°C, and the heating effect was maintained for 3 seconds. The volatile fragments were ionised by a low energy electron beam of approximately 25 eV. Ionised fragments were driven towards the quadruple by a negatively charged repeller plate. The mass ion counts were collected and stored on the hard disk of a microcomputer as the mass ion spectrum for each sample (25).

**Data analysis:** The raw data was processed and analysed by using the PYMENU program (Horizon Instrument Ltd.) and the GENSTAT V statistical package (26) on an IBM PC computer. Normalised data sets were analysed by principal components analysis. Plots of the first two or three principal components were produced as plots of the spectral scores, the position of the pyrolysis spectra on the principal component axes. A plot of the mass loadings for the axes gave information about the contribution of masses to the principal component axes. Canonical variate analyses of all of the principal components accounting for more than 0.5% of the total variance was carried out to give combined principal component-canonical variate analysis (PC-CVA). The data from PC-CVA were plotted as Mahalonobis distances (27). The Mahalonobis distance matrix was standardised by dividing the maximum inter-group distance and then converted to a similarity matrix (28). The values in the similarity matrix were examined using the unweighted pair grouping method (UPGMA) with arithmetic averages algorithm (29).

**Results and Discussion**

Pyrolysis mass spectrometry was performed successfully and different clusters were obtained from different samples of *Rhodococcus ruber* in the ordination diagram. PCCV analysis of Pyrolysis Mass Spectra of biomass from batch, carbon-limited, phosphate-limited, and magnesium-limited cultures is shown in Figure 2, and the growth conditions for *Rhodococcus ruber* in batch and continuous cultures related to Figure 2 are also given in the Table 1.

![Figure 2: PCCV analysis of Pyrolysis Mass Spectra of biomass from batch, carbon-limited, phosphate-limited, and magnesium-limited cultures.](image-url)
Some typical pyrolysis mass spectra are shown in Figure 3. It is evident from these representative data that most of the spectra fall within the mass ranging from 51 to 140 m/z, with only very low intensity ions found above this mass range. It is also clear from these pyrograms that spectra are qualitatively similar.

In an initial analysis, the batch grown cells showed a significantly different composition from that of the continuous culture. All batch grown samples from different stages of the growth cycle are clustered together in the ordination diagram. Large differences were obtained between early exponential phase culture and stationary phase culture. In contrast, little variation was found between samples taken during the late exponential phase and the early exponential phase (Fig. 2).
As can be seen in Figure 2, all carbon-limited cultures are clustered together. Although there were large variations between samples such as CL2 and CL7, much less variation was observed between CL3 and CL4, and CL6 and CL7. All phosphate-limited samples are clustered together in the PCCV ordination diagram in Figure 2. All the phosphate-limited samples are similar but the variations in the growth conditions between samples did give rise to differences between the samples.

Figure 3. Samples of pyrolysis mass spectra derived from batch culture and continuous culture.
Only one sample of magnesium-limited cell was analysed and it is shown in Figure 2. It can be seen that the magnesium-limited biomass is separated from the rest. It is notable that large differences were obtained between magnesium-limited cells and carbon-limited cells.

Pyrolysis mass spectrometry is a sensitive, discriminatory technique for comparing the macromolecular composition of biomass samples. It has been used to detect the presence of specific components in cell samples in *Escherichia coli* (19, 22).

In the present study, we hoped to use PyMS to map the changes in *Rhodococcus ruber* with different growth conditions. Indeed, we found that the composition of the cells did differ depending upon the nature of the growth environment (Fig. 4). The next major factor to influence the pyrolysis mass spectra, and the overall composition of the organism, was growth rate (Fig. 4). However, neither temperature nor pH was seen to have a clearly interpretable effect upon the pyrolysis mass spectra.

The effect of growth rate is shown most clearly in the carbon-limited cultures with a shift in composition reflected by a change of position of the samples in the ordination diagram from left to right and up. A similar left to right trend was observed for phosphate-limited cultures (Fig. 4).

With only a limited range of samples, and given the black box nature of the pyrolysis spectra, it was not possible to determine which cellular components differed between samples. With a wider range of samples, for which fatty acid and lipid analyses were available, it might have been possible to train a neural network to learn the relationship between the pyrolysis spectra and the lipid composition. It would then have been possible to analyse many samples relatively quickly. However, given the variable nature of growth in the chemostat with this organism, it was not possible to generate large numbers of samples with well-defined growth conditions for such analysis.

![Figure 4](image-url) The effect of growth rate on the pyrolysis mass spectra of *Rhodococcus ruber* grown under carbon, phosphate and magnesium limitation.
In conclusion, the PyMS data for cells of Rhodococcus ruber depends on whether the organism was grown in batch or continuous culture and the growth limitation for the latter. Growth rate had only a small effect, whereas pH and temperature gave no clear trends.

The most significant feature of the results from batch cultures was the clear distinction of stationary cells from exponential phase bacteria (Fig. 2), demonstrating some significant chemical changes in resting cells. Cells from carbon, phosphate and magnesium limitation were distinguished from each other and from batch culture organisms (Fig. 2).

References


