J. Microbiol. Biotechnol. (1998), **8**(5), 523 - 530

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Rapid and Quantitative Analysis of Clavulanic Acid Production by the Combination of Pyrolysis Mass Spectrometry and Artificial Neural Network

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Received: September 15, 1998

Abstract Rapid and quantitative analysis of physiological change and clavulanic acid production was studied by the combination of pyrolysis mass spectrometry (PyMS) and artificial neural network (ANN) in *Streptomyces clavuligerus*. Firstly, the continuous culture studies were carried out to get the physiological background and PyMS samples. Clavulanic acid production was inversely related to growth rate: Mycelium growth and q_{cla} were optimum at 0.1 h^{-1} and 0.025 h^{-1}, respectively. Changes in specific nutrient uptake rates (q_{gly} and q_{amn}) also affected clavulanic acid production since clavulanic acid production appeared to be stimulated by the limitation of carbon and nitrogen. Fermentation broth containing mycelium taken from continuous cultures was analyzed by PyMS, and the PyMS spectra were analyzed with multivariate statistics. PCCV plots revealed that samples harvested under the same culture condition were clustered together but samples from different culture conditions formed separate clusters. To deconvolute the pyrolysis mass spectra so as to obtain quantitative information on the concentration of clavulanic acid, ANN was trained on PyMS data using a radial basis function classifier. The results showed that the physiological stages with different growth rate were successfully differentiated and it was possible to monitor the clavulanic acid production precisely and rapidly.

Key words: Clavulanic acid, *Streptomyces clavuligerus*, pyrolysis mass spectrometry, artificial neural networks

Streptomycetes are of interest because of their ability to produce various unique metabolites (physiological differentiation). The onset of the metabolite production is always accompanied with morphological differentiation that is characterized by the formation of a substrate mycelium, an aerial mycelium and arthrospores [3]. Since it is not easy to determine the physiological differentiation state quantitatively, quick and precise methods for the determination of differentiation would be very useful for the optimization of the metabolite production.

Pyrolysis mass spectrometry (PyMS) has been used as a useful technique for the analysis of complex organic materials, natural biological products [7, 8, 19, 20], and for the rapid and quantitative analysis of bioprocesses [7, 8]. The method is rapid (the typical sample time is less than 2 min) and can be automated. We therefore considered that PyMS might be a suitable method for monitoring the physiological condition and secondary metabolite production. Pyrolysis is the thermal degradation of a complex material in a vacuum. It causes molecules to cleave at their weakest point to produce smaller, volatile fragments - the pyrolysate [11]. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass to charge ratio (m/z) to produce a pyrolysis mass spectrum.

The technique produces large amounts of complex experimental data in the form of mass spectra which qualitatively and quantitatively represent the initial sample composition. Therefore, suitable numerical methods should be employed to understand the complex spectra, and principal component analysis (PCA) and canonical variate analysis (CVA) are two multivariate statistical techniques which are commonly used in the analysis of such data. Recently, to deconvolute the PyMS spectra, artificial neural networks (ANNs) are an increasingly well known means of uncovering complex, nonlinear relationships in multivariate data, while still being able to map the linear relationship. ANNs can be considered as collections of very simple computational units which can take a numerical input and transform it into specific output. Provided the ANN is trained on representative

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data from the system analyzed, like PyMS data from microbial samples, it can produce a generalized solution. Their application is well researched and new uses are being developed all the time [14]. The combination of PyMS and ANNs has proved to be very powerful in the identification of microorganisms [4, 6] and in rapid screening and the quantitative analysis of metabolite that has been overproduced in fermentor broths [9, 10].

In this work, the effects of growth rate and nutrient feed rates (carbon or nitrogen) on clavulanic acid production in *Streptomyces clavuligerus* were evaluated and the physiological differentiation of the culture were analyzed by the combination of PyMS and ANNs.

MATERIALS AND METHODS

Microorganism and Media

Streptomyces clavuligerus ATCC 27064 was used. Stock culture medium consisted of (w/v): 1% glucose, 0.2% peptone, 0.3% yeast extract, 0.1% beef extract and 1.8% agar. Seed culture medium consisted of (w/v): 1% glycerol and 1% yeast extract. The carbon limited medium for the continuous culture consisted of (w/v); 0.4% glycerol, 0.1% NH_4Cl, 0.2% KH_2PO_4, 0.005% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$ and 0.005% $MnCl_2 \cdot 4H_2O$, $ZnCl_2$ 0.005%. In the nitrogen-limited culture, 0.7% glycerol and 0.05% NH_4Cl were used as carbon and nitrogen sources. The initial pH of the media was adjusted to 7.0 before steam sterilization. Phosphate and other salts were separately sterilized by membrane filtration (0.2 μm, Millipore, U.S.A.) and added asceptically.

Strain Maintenance and Culture Conditions

Strains were maintained by transfer to slopes of the stock culture medium each month, and were stored at 4°C. For solid cultures, one loopful of spores were inoculated evenly on the main agar culture medium and incubated at 28°C. For submerged culture, spores developed on the stock culture medium were inoculated to 100 ml of the seed culture medium and incubated at 28°C for 2 days. The seed culture (300 ml) was inoculated to 3 l of the main culture medium contained in a jar fermentor (Model KF-5L, Korea Fermentor Co.). The culture temperature was maintained at 28°C and the initial pH was controlled at 7.0. Agitation and aeration were 300 rpm and 0.5 vvm, respectively. For a continuous culture, the seed culture (150 ml) was inoculated to 1.5 l of the main culture medium, and the carbon or nitrogen limited continuous medium started to be fed after 2 days.

Analytical Methods

Morphological characteristics were observed with a phase-contrast microscope (Nikon Laphot). To measure mycelium growth, mycelium was collected by vacuum filtration (Whatman GF/C paper), dried at 80°C for 24 h, and weighed. Clavulanic acid was determined using HPLC as described by Foulstone and Reading [5]. The culture supernatant was prepared by ultrafiltration (cut-off 5000, Amicon, U.S.A.). The filtrates were treated with imidazole reagent at room temperature for 20 min. The analysis was performed with a C_{18} μbondapak column (Waters, U.S.A.), and the effluents were detected at 312 nm with an UV detector (Gilson). Potassium clavulanate (Worthing, U.K.) was used as the standard.

The concentrations of glycerol and sucrose were measured by HPLC with an Aminex HPX-87H cation exchange column (Bio-Rad, Richmond, U.S.A.), using an effluent of 0.005 N H_2SO_4 at 30°C. Effluents were detected with an RI (Refractive Index) detector and quantified with a Bio-Rad 3392A integrator. All peaks were compared with those produced by authentic standard compounds.

Fermentation Kinetic Parameters

Various kinetic parameters were analyzed with the data obtained from continuous cultures as suggested by Pirt [17]. $\mu = D$, where μ is the specific mycelium growth rate, D is the dilution rate (h^{-1}); $q_{gly} = D\ (s_o - \bar{s}\ ) / \bar{x}$, where q_{gly} is specific glycerol uptake rate, s_o is the concentration of glycerol in the feed medium, $\bar{s}$ is the steady state concentration of glycerol (g l^{-1}), and $\bar{x}$ is the steady state concentration of biomass (g l^{-1}), respectively; $q_{amn} = D\ (n_o - \bar{n}) / \bar{x}$, where q_{amn} is the specific ammonium ion uptake rate, n_o is the concentration of ammonium ions in the feed medium, $\bar{n}$ is the steady state concentration of ammonium ions (g l^{-1}), respectively; $q_{cla} = D\ \overline{cla} / \bar{x}$, where q_{cla} is the specific clavulanic formation rate, $\overline{cla}$ is the steady state value of clavulanic acid; $Y_{cla/x} = \overline{cla} / \bar{x}$; $Y_{x/gly} = \bar{x} / (s_o - \bar{s})$.

Pyrolysis Mass Spectrometry

Samples (1 ml) were taken from the continuous cultures and lyophilized, and then the samples were resuspended with 0.1 ml saline solution (0.8%, w/v) before loading into PyMS foil. Clean iron nickel foils (Horizon Instruments, U.K.) were inserted into clean pyrolysis tubes, such that 5~6 mm was protruding from the mouth of the tube. 2 μl of the resuspended samples was pipetted onto 530°C PyMS foils (Horizon Instruments), and dried at 80°C for 10 min, and then the foils were pushed into the tube using a stainless steel depth gauge. Each culture sample was prepared in triplicate, and was then pyrolized on the RAPyD 400 (Horizon Instruments).

The sample tube carrying the foil was heated, prior to pyrolysis, at 100°C for 5 s. Curie-point pyrolysis was at 530°C for 3 s, and the pyrolysate entered a gold plated expansion chamber heated to 150°C, whence diffused down a molecular beam tube to the ionization chamber

of the mass spectrometer. To minimize secondary fragmentation of the pyrolysate, the ionization method was low voltage electron impact ionization (25 eV). Non-ionized molecules were deposited on a cold trap, cooled by liquid nitrogen. The ionized fragments were focused by the electrostatic lens of a set of source electrodes, accelerated and directed into a quadrupole mass filter. The ions were separated by the quadrupole, on the basis of their mass to charge ratio, detected and amplified with an electron multiplier. The mass spectrometer scans the ionized pyrolysate 160 times at 0.2 s over the m/z range of 51 to 200, in one tenth of a mass unit interval. These were then integrated to give the unit mass. The data were collected by an online computer.

Multivariate statistical analysis was carried out using RAPyD software and GENSTAT [16]. The data were normalized as a percentage of total ion count to remove the influence of sample size. The first stage was the reduction of the data by principal component analysis (PCA), which is a well known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. Data were reduced by keeping only those principal components (PCs) whose eigenvalues accounted for more than 0.1% of the total variance. Canonical variates analysis (CVA) then separated the samples into groups on the basis of the retained PCs and the knowledge of the appropriate number of groupings [13]. A regression using PCs or PCCVs against clavulanic acid concentration was performed.

The Artificial Neural Networks

The ANN algorithm used in this work was radial basis function [1, 2, 12, 15, 18]. The ANN was programmed and modified in Matlab using the neural network toolbox. The input vector consists of 150 values which represent the mass/charge ratio (m/z) counts for each molecular weight in the range of 51 to 200 daltons. The spectra were each normalized to their total ion count, and these values were used as the input vectors. The output values were encoded with clavulanic acid concentrations of various continuous cultures. The PyMS data was divided into training, validation, and testing data sets. The success of training was determined with the average sum square value between desired output vector and the predicted value [$1/n*\Sigma$(desired output vector - predicted value)^2], and the final error goal was set to 0.01. During the training, validation was performed with the validation data set to monitor training and avoid overtraining of the neural networks.

Radial Basis Classifier (RBF)

The RBF architecture consisted of three layers, each layer connected to the next in a feedforward manner. The input layer is nominal in the sense that it merely passes the values forward to the next layer. The values received by the hidden layer are given by ($\|x - c_i\|$) where x is the input matrix and c_i is the value at the center of each radial node. The new values are then passed through each Gaussian node $\phi(\|x - c_i\|)$ as a kernel function. Associated with the hidden to output connections are conventional signal multipliers: the final output processing unit merely yields a weighted sum of its inputs (1'). The Gaussian/kernel function is given by (2').

$$f(x) = \sum_{i=1}^{n} w_i \phi \| x - c_i \| \quad (1')$$

$$\phi \| x - c_i \| = 1/\exp[b(x - c_i)^T * (x - c_i)]^{1/2} \quad (2')$$

where b is the system parameter which determines the steepness of each kernel function. Adjustment of the weights and bias was determined by minimizing the error.

RESULTS AND DISCUSSION

Growth and Clavulanic Acid Production in Continuous Cultures

Carbon limited continuous cultures of *S. clavuligerus* were first used to assess the effect of growth rate on the production of clavulanic acid. The steady-state data for the biomass, glycerol, ammonium ion, and clavulanic acid production in the continuous culture are shown in Fig. 1. The culture behaved as carbon-limited condition

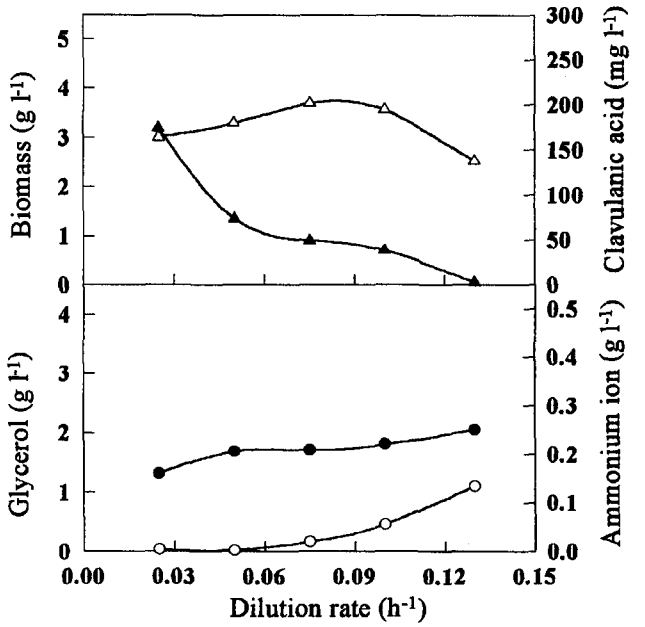

Fig. 1. Changes of (○) glycerol, (●) ammonium ion, (△) biomass and (▲) clavulanic acid concentrations in the continuous culture of *S. clavuligerus*.

below 0.075 h^{-1}. The steady-state values of biomass slightly increased as the dilution rate increased, but those of clavulanic acid were inversely related to growth rate.

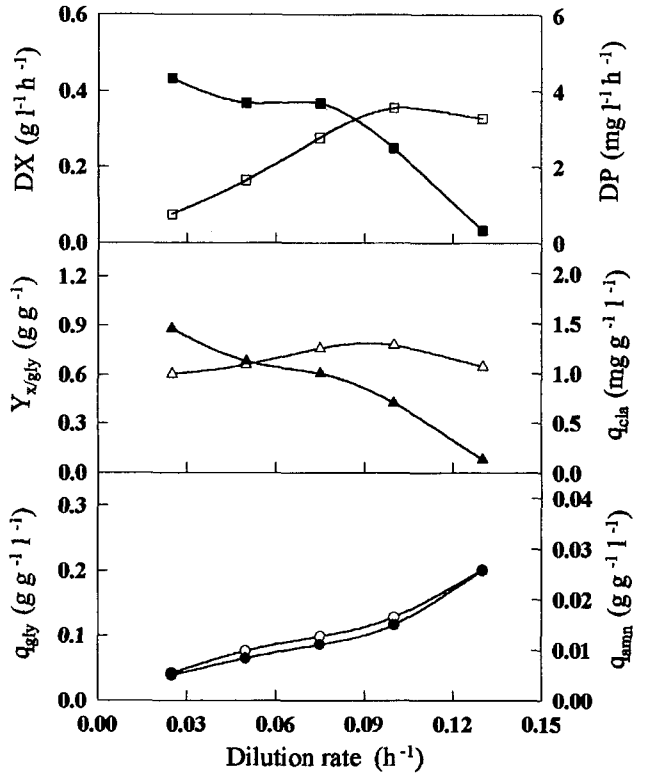

Fig. 2. Kinetic parameters of steady states in the continuous culture of *S. clavuligerus*. (○) q_{gly}, (●) q_{amn}, (△) $Y_{x/gly}$, (▲) q_{cla}, (□) *DX*, and (■) *DP*.

The kinetic parameters calculated from the steady-state values are shown in Fig. 2. The specific uptake rates of glycerol and ammonium ion (q_{gly} and q_{amn}) increased with the specific growth rate. However, the specific production rate of clavulanic acid (q_{cla}) decreased as growth rate increased, 0.025 h^{-1} being considered to be optimum. These data indicated that the optimum condition for the clavulanic acid was different from that of mycelium growth.

Nutrient Uptake Rate and Clavulanic Acid Production

In order to distinguish the effect of the growth rate from the nutrient uptake rates on clavulanic acid production, a continuous culture was carried out at a fixed dilution rate (0.04 h^{-1}) where the feed concentration of glycerol or ammonium ions was varied. The steady-state values of biomass and clavulanic acid concentration varied with the feed concentration of glycerol (Fig. 3A). The kinetic parameters calculated from the steady-state values showed that biomass was related to glycerol feed rate while clavulanic acid production and q_{cla} was inversely related to the feed rate (Fig. 3A).

Continuous cultures, where the in-flow concentration of ammonium ion was varied at a fixed dilution rate of 0.04 h^{-1}, were also carried out (Fig. 3B). The steady-state values of biomass increased with the feed nitrogen concentration but those of clavulanic acid decreased. The clavulanic acid production was optimum at a feed ammonium concentration of 0.005 g $l^{-1}h^{-1}$, and was repressed at higher uptake rates (Fig. 3B). From the results, it was evident that concentrations of glycerol and ammonium played an important role on clavulanic acid

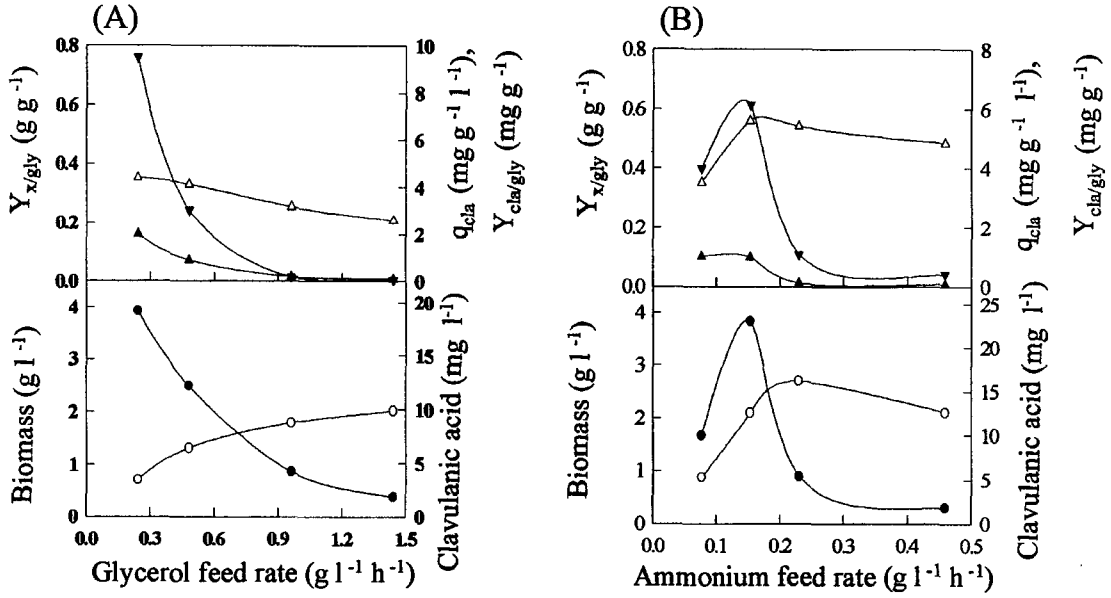

Fig. 3. Kinetic parameters of steady states with (A) feed glycerol concentration and (B) feed ammonium concentration at a fixed dilution rate (D=0.04 h^{-1}). (○) q_{gly}, (●) q_{amn}, (△) $Y_{x/gly}$, (▲) q_{cla}, and (▼) $Y_{cla/gly}$.

production: ammonium ions were more critical to give inhibitory effect at feed rates higher than 0.005 g $l^{-1}h^{-1}$.

Analysis of Physiological States in the Continuous Culture by PyMS and Multivariate Statistics

In order to evaluate the physiological state of fermentation and clavulanic acid production quantitatively and rapidly, whole culture broths of *S. clavuligerus* were taken from steady states of different dilution rates, and then the whole broths were analysed by PyMS. The PyMS spectra were very complex and varied upon the dilution rates (Fig. 4). It indicated that components of each sample were not identical, but changed with the dilution rate. Figure 4C shows a difference spectrum between the normalized averages of low dilution rate and those of high dilution rate. The spectrum indicates that m/z 52, 53, 54, 55, 58, 59, 64, 65 and 68 were correlated with low dilution rate state while m/z 61, 62, 63 and 74 were related to high dilution rate state.

Since some masses appeared to be correlated with physiological differences, all the m/z in the range of 51 to 200 were plotted against dilution rate in order to see whether the PyMS spectra have suitable information on the physiological differentiation and to select masses more suitable. This was achieved by presenting triplicate pyrolysis mass spectra of one mass, and then the plot was compared with clavulanic acid production at each dilution rate (Fig. 5). The m/z 52, 58, 64, 66 and 68 show the best match with the clavulanic acid concentration. Interestingly, the regression of m/z 59, 61 and 62 was not successful although the difference spectrum between spectra from low dilution rate and high dilution rate show that ion count values for those masses have a notably large peak in the subtraction spectrum (Figs. 4, 5).

The results indicate that the pyrolysis mass spectra can be used to monitor the physiological differences and clavulanic acid production. The physiological differences

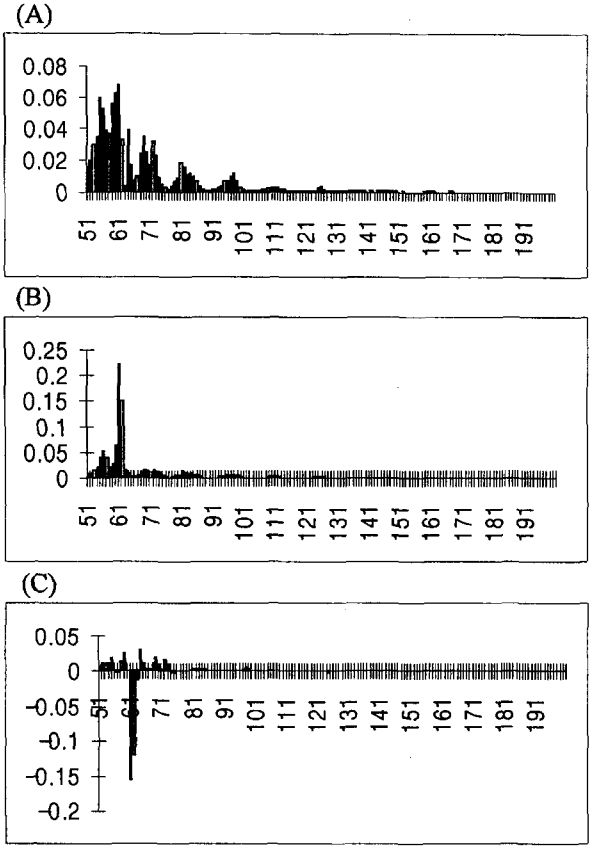

Fig. 4. Normalized pyrolysis-mass spectra of (A), low dilution rate state; (B), high dilution rate state; (C), the difference spectrum between the normalized average of the pyrolysis spectra of (A) and (B). X axis is m/z values and Y axis is normalized ion counts, respectively.

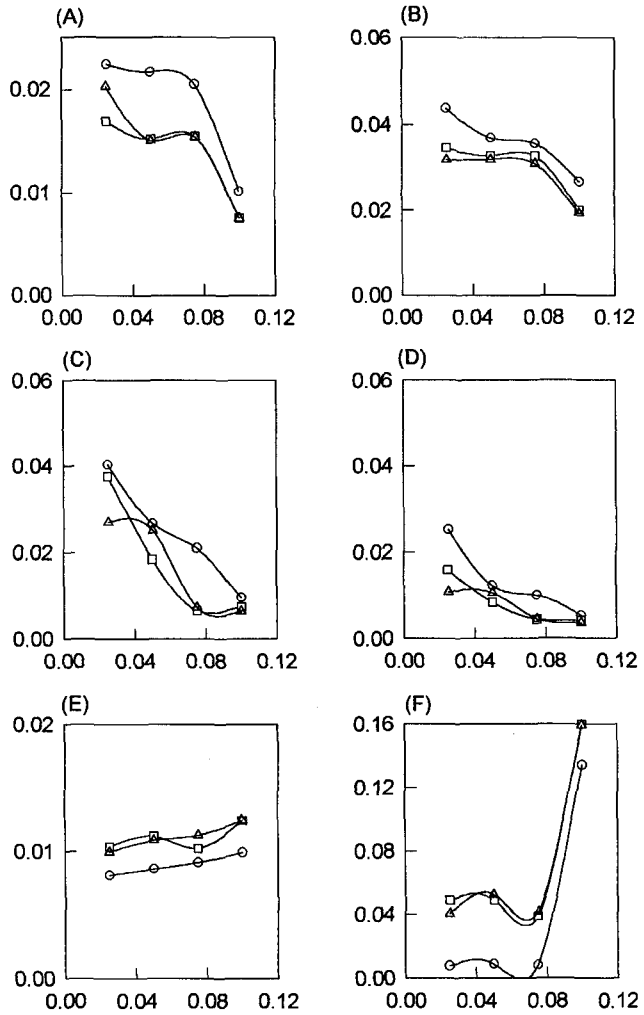

Fig. 5. The plots of the ion counts for the m/z values correlated with clavulanic acid production against dilution rate.

Three sets of samples are shown in the figures. X axis is dilution rate and Y axis is normalized ion counts, respectively. The m/z values are (A), 52; (B), 58; (C), 64; (D), 68; (E), 62; (F), 74.

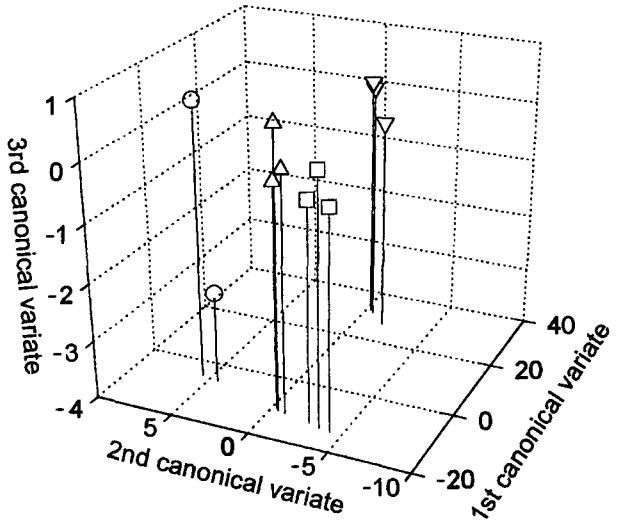

Fig. 6. PCCV plot of samples taken from steady states of various dilution rates. (○) 0.025 h^{-1}, (△) 0.05 h^{-1}, (□) 0.075 h^{-1}, and (▽) 0.1 h^{-1}.

involved in clavulanic acid production might cause the difference in the PyMS spectra. In this respect, numerical methods were employed to understand the complex PyMS spectra to analyze the physiological differences and clavulanic acid production.

The normalized PyMS data in the range of 51 to 200 m/z were statistically analyzed with multivariate statistics techniques including PCA and CVA so as to discriminate the physiological differences of *Streptomyces clavuligerus*. At the first stage, the PyMS data were reduced by keeping only those principal components (PCs) whose eigenvalues accounted for more than 0.1% of the total variance. Five PCs more than 0.1% of total variance were selected. CVA then separated the samples into groups on the basis of the 5 PCs. As a result, the PCCV plot shows that the variates of one steady state can be separable from those of the others (Fig. 6). From the analysis, it was evident that the sample obtained from one condition could be clearly distinguished from the other samples of different conditions by PyMS, and the effects of the dilution rate on the cellular component could also be identified.

Monitoring the Clavulanic Acid Production by PyMS and ANN

For deconvolution of the pyrolysis mass spectra, artificial neural networks were developed and optimized. Artificial neural networks (ANNs) can give a generalized solution of the relationship between complex phenomena such as pyrolysis spectrum, and may provide considerable advantages in prediction against different biological backgrounds [4, 9, 10].

A feedforward ANN, radial basis classifier (RBF) which uses a Gaussian function as the transfer function in the hidden layer was trained with PyMS data. The PyMS spectra taken from various dilution rate conditions were each normalized to their total ion count, and 3 sets of data were divided into training, validation, and testing data sets, which in turn were used as the input vectors. The output values were encoded with clavulanic acid concentrations. The steepness of the Gaussian function varied from 0.05 to 0.6, and the number of nodes in the hidden layer also varied from 1 to 7. The final output processing unit merely yielded a weighted sum of the hidden layer output. As a result, the optimized structure of RBF was determined according to the SSE values of training and validation sets, and the optimum value for the steepness and the number of nodes was determined to be 0.1 and 5, respectively (Fig. 7). The final error was 0.001, and the training time was much shorter than the backpropagation network (data not shown). The estimated values of input vectors of training, validation, and test sets are shown in Fig. 8. The result indicates that clavulanic acid concentration in the whole culture broth can be estimated rapidly and accurately by the combination of PyMS and ANN.

The whole samples taken from the steady state in the various ammonium feed rates (Fig. 3) were also analyzed by PyMS, and the clavulanic acid concentration of the samples was estimated by the trained neural network from Fig. 7 (Table 1). The radial basis classifier gave the reliable prediction of clavulanic acid production of samples with different background. The estimated values indicate that the combination of PyMS and artificial neural network can provide the possibility to monitor the differentiation state quantitatively in *Streptomyces clavuligerus*.

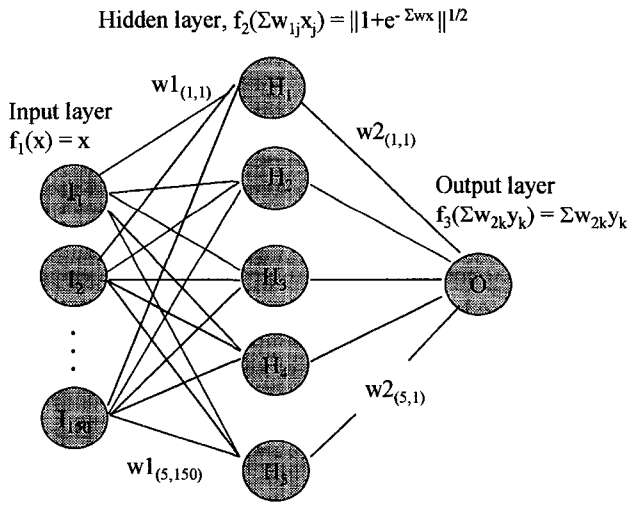

Fig. 7. A radial basis classifier consisting of 150 inputs and one output connected to each other by one hidden layer consisting of 5 nodes.

In the architecture shown, adjacent layers of the network are fully interconnected.

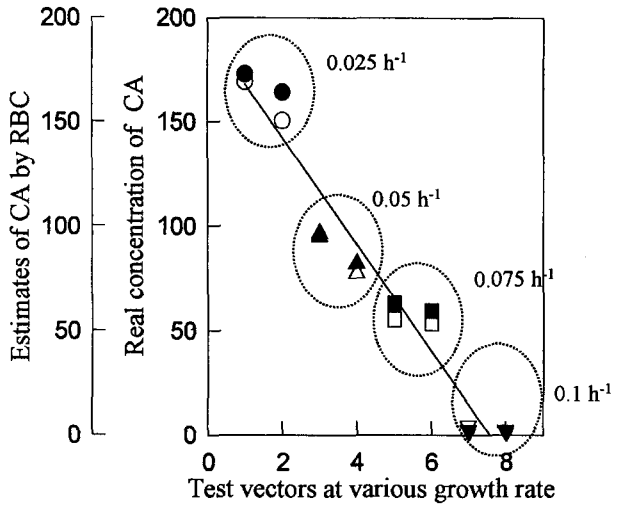

Fig. 8. The result of RBC training with whole cell pyrolysis mass spectra.

The hollow symbols are real concentration of clavulanic acid at (○) 0.025 h^{-1}, (△) 0.05 h^{-1}, (□) 0.075 h^{-1}, and (▽) 0.1 h^{-1}. The filled symbols are estimates by trained RBC at (●) 0.025 h^{-1}, (▲) 0.05 h^{-1}, (■) 0.075 h^{-1}, and (▼) 0.1 h^{-1}.

Table 1. The estimation of clavulanic acid production by trained RBC in *Streptomyces clavuligerus*.

	Estimates of transient samples			
	1[a]	2[b]	3[c]	4[d]
Real estimation	10	23	3.34	1.87
Estimation by RBC	7.6	27	5.2	1

[a,b,c,d]The whole culture broths containing mycelium were taken from the steady states of various ammonium feed rates (0.0025, 0.005, 0.01 and 0.02 g $l^{-1}h^{-1}$, respectively), and then pyrolyzed. The clavulanic acid concentration was estimated by trained RBC.

We have shown that the pyrolysis mass with multivariate calibration and ANNs can analyze the differentiation stage qualitatively and quantitatively in *Streptomyces clavuligerus*. Changes in culture conditions such as nutrient starvation triggered physiological differentiation followed by morphological differentiation in *Streptomyces* spp. The differentiation gave rise to increase culture viscosity, from which imperfect mixing and lowering in antibiotic production resulted. Therefore, rapid and precise methods for the determination of physiological differentiation are necessary for the monitoring of fermentation processes, and determining the physiological conditions.

In this work, the effect of dilution rate, and nutrient concentration in the feed (carbon or nitrogen) on clavulanic acid production in *Streptomyces clavuligerus* were evaluated and the physiological differentiation of the culture were analyzed by applying PyMS and ANNs. The combination of PyMS with multivariate and ANN was proved to be a method for the rapid and quantitative analysis of the state of physiological differentiation such as in the analysis of clavulanic acid production in *S. clavuligerus*.

Abbreviations: ANN, artificial neural network; CVA, canonical variate analysis; PCA, principal components analysis; PyMS, pyrolysis mass spectrometry; PCs, principal components; PCCV, principal component-canonical variate; RBF, radial basis function; q_{gly}, specific glycerol uptake rate; q_{amn}, specific ammonium uptake rate; q_{cla}, specific clavulanic production rate; $Y_{x/gly}$, growth yield coefficient; *DX*, cellular productivity; *DP*, clavulanic acid productivity

Acknowledgments

This work was partially supported by an international research grant (BBSRC) sponsored by the Korea Science and Engineering Foundation (KOSEF). It was also partially supported by a research grant from the Research Centre for Molecular Microbiology (RCMM) sponsored by the Korea Science and Engineering Foundation (KOSEF). We thank Prof. M. Goodfellow for the support.

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