Journal of the Research Institute of Industrial Technology, Vol. 18, 2001

# Analyses of Recycling Oxic and Anoxic Swine Wastewater Treatment System Using Neural Networks and Molecular Techniques

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# 신경회로망과 분자생물학적 기법을 이용한 순환식 호기성 및 미호기성 돈분폐수 처리 시스템의 분석

최 정 혜, 손 준 일, 양 현 숙, 정 영 륜, 이 민 호, 고 성 철

## 요 약

본 논문에서는 신경회로망을 이용하여 순환식 돈분처리 시스템의 실시간 모니터링을 궁극적으로 구현할 수 있는 새로운 방법을 제안하였다. 즉 미생물 군집내의 개체군밀도에 따른 각 처리조(유입수, 발효조, 폭기조, 1차 침전조 및 4차 침전조)에서의 폐수처리 과정을 모델을 시도하였다. 측정 데이터에 대해 우선 principal component analysis(PCA) 분석을 적용하여 각 처리조에서의 입력(미생물 밀도와 부유물질, COD, 암모니아성 질소, 무기인, 총인과 같은 처리요소)과 출력간의 상관관계를 파악하고, 각각의 처리조마다 독립된 신경회로망을 적용하여 폐수처리 과정을 모델링하였다. 신경회로망의 입력으로 현재 탱크에서의 미생물의 개체군밀도를 직접 이용하는 대신 PCA 분석 결과를 이용함으로써, 비교적 적은 수의 데이터로 효과적인 모니터링 시스템을 구현할 수 있었다. 즉 각 처리조별로 학습된 신경회로망들을 연결하여 분석한 결과 2일 동안의 폐수 처리 변화를 비교적 정확히 예측할 수 있었다. 또한 분자생물학적 기법 (PCR)을 이용하여 시스템 내에서 분리된 타가영양 박테리아로부터 암모니아 흡수에 관여된 glutamine synthetase (GS) 유전자의 존재를 확인하였다.

**Key words** : piggery slurry; neural network; principal component analysis (PCA); lactic acid bacteria (LAB); *Alcaligenes faecalis*; glutamine synthetase (GS)

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# 1. Introduction

Swine wastes may cause a serious degradation of water quality such as eutrophication and spread of pathogens in water bodies (*i.e.*, lakes, rivers and groundwater as water supply sources)[1]. The daily volume of livestock wastewater in Korea reached 197,000$m^3$, and 50% of the volume was generated from dairy farms that were not target for a legal pollution control. The amount of wastewater is relatively small compared with total wastewater including industrial and domestic wastewater (7% of the total), but contributes significantly to the pollution of the receiving waters because of its high organic nutrient concentration (>BOD 20,000 mg/L) [2]. While an activated sludge system has been proven to be effective in the treatment of piggery slurry at large scale farms (more than 1,000 heads), the system may not ensure the effect in small or middle scale farms (less than 1,000 heads) in terms of its operation cost.

Recently a recycling reactor system operated under sequential oxic and anoxic conditions for the swine wastewater has been developed, in which piggery slurry is fermentatively and aerobically treated and then the partial effluent recycled to the pigsty [3]. This system appears to significantly remove offensive smells (at both pigsty and treatment plant) and BOD, and turns out to be cost effective for relatively small-scale farms.

One of the best known models applied for wastewater treatment system so far is the activated sludge model NO. 1(ASM 1) introduced by International Association for Water Quality (IAWQ) [4]. Application of the model to field treatment system, however, may have some limitations because there are many operational parameters and has quite variable kinetic characteristics within the treatment system over time [5].

On the other hand, neural network models that imitate the functions of our human brain have been successfully used to resolve many engineering problems such as complex pattern classification and control of highly nonlinear dynamic systems [6-9]. Those models have the characteristics of massive parallelism, many degrees of freedom, and adaptive learning. It was recently well known that the multi-layer neural networks can approximate a function in $L^p$ within an arbitrary accuracy [10], and can generalize a new data set that was not used in the learning process [11]. Recently a progress has been made in application of neural networks to control biological and chemical engineering processes. There has been, however, no report dealing with a neural network modeling for biological swine wastewater treatment system.

This study was carried out to elucidate mechanism of the recycling piggery slurry treatment system using such as variables population dynamics, activity of heterotrophic bacteria, and treatment effects based upon suspended solids (SS), total nitrogen (T-N), ammonia nitrogen ($NH_4^+$-N), total phosphorus (T-P), *ortho*-phosphorus (*o*-P) and chemical oxygen demand (COD) as input or output variables. These variables were used to establish a non-linear model emulator using multi-level neural networks that could eventually allow real time monitoring and prediction of the treatment system. We also tried to elucidate a mechanism for ammonium removal using molecular biological techniques.

## 2. Materials and methods

### 2.1. System Overview

A scheme for the recycling treatment system at a bench scale is shown in Fig. 1. Detail description of the reactor operation has been shown in the previous report [12]. Piggery slurry and treated effluent used as washing water were collected in tank 1, and this influent then flows into the fermentation tank (tank 2; 15L). There is a channel between tank 2 and an aeration tank (tank 3; 15L) so that the fermented wastewater can be transported into tank 3 where oxidative treatment occurs under aeration condition (7.8v/v/m). The treated water then goes through sedimentation process in tanks 6 and 7, and finally is stored in tank 8. A portion of the effluent was recycled and used to wash the pigsty.

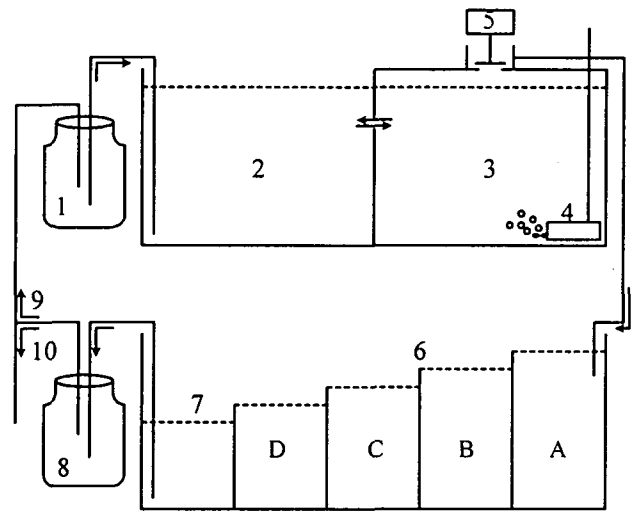


**Fig. 1. Schematic diagram of the recycling treatment system for piggery slurry. 1 Influent tank; 2 Fermentation tank; 3 Aeration tank; 4 Blower; 5 antifoaming device; 6 Sedimentation tank (A, B, C and D); 7 Reservoir; 8 Storage tank; 9 Recycling flow; 10 for land application as a fertilizer.**

The wastewater used in this study was sampled from a mixing and storage tank at Kimhae Piggery Slurry Treatment Plant (Kimhae, Kyungnam, Korea) and had a COD (*ca.* 4000 mg/L), BOD (*ca.* 7000 mg/L), T-N (*ca.* 2100 mg/L), and T-P (*ca.* 172 mg/L). The influent consisted of piggery slurry (33% v/v), effluent (57%) and tap water (10%) and was supplied every 4 days. Glucose was added to the formulated influent to make a C/N ratio of 100:15 [13] and a microbial agent (YC2000, Yoonchang Agricultural Management, Inc., Cheju) was also added up to 1 %(w/v). The hydraulic retention time of the system was 4 days and was operated for 47 days.

### 2.2. Isolation, Identification and Quantification of Microorganisms

Heterotrophic bacteria potentially involved in the piggery slurry treatment within the system were isolated using the appropriate media [14]. To isolate and grow lactic acid bacteria (LAB), MRS medium was used. LAB were grown at least 2 weeks before

identification and counting were performed. Other heterotrophs were grown on TSA (Trypticase Soy Agar, Difco) for at least 1 week, and then identified and counted.

The bacterial communities in the system were analyzed based on their isolation, identification and determining the colony forming unit number (population density) of dominant populations in each medium. Identification of the bacteria was performed based on cultural, physiological and biochemical characteristic described by Smibert et al. [15] and Holt et al. [16]. Utilizations of sugar, amino acids and organic acids by Gram-negative bacteria were tested using an API Kit (bio Merieux sa, France) according to the manufactures protocol.

### 2.3. Analysis of Piggery Slurry Samples from the Treatment System

Monitoring parameters, such as SS, T-N, $NH_4^+$-N, T-P, *o*-P and COD, were measured for piggery slurry samples taken daily following Standard Methods for the Examination of Water and Wastewater [17]: COD by closed reflux, titrimetric method, T-P and *ortho*-P by ascorbic acid method, suspended solids by total suspended solids cride method, and $NH_4^+$-N by indol phenol method.

### 2.4. Modeling of the Treatment System Using Neural Networks

For an optimal treatment of piggery slurry, it is important to understand the physiological characteristics of microorganisms and their relationships, but may be difficult to identify the relationship by a linear analytical method. The relationship between population densities of the microorganisms and the treatment efficiency shows a nonlinear dynamic characteristic. We used a multi-layer neural network with an error back propagation algorithm to model the complex relationship in the recycling system. Since the multi-layer neural network is able to approximate an arbitrary nonlinear function with sufficient input and output data, the modeling of the recycling piggery slurry treatment system can be accomplished using the neural network for complex dynamic systems. For modeling of the recycling system, we considered cause and effect relation in each tank. As independent parameters in each tank, the population densities of heterotrophic microorganisms MRS-1, TSA-1, TSA-2, and TSA-3 were considered because those could dominantly affect the piggery slurry treatment efficiency. Also, COD, total-P, *ortho*-P, SS and $NH_4^+$-N were considered as treatment parameters in each tank. Thus, we designed a multi-layer neural network in which the input nodes consisted of 4 independent parameters in the current tank and 5 treatment results in previous tank, and the output nodes generated the 5 treatment results in the current tank.

To model the recycling system, there are two ways to use the neural network. One is to use a single neural network for modeling the characteristic of all the tanks in the recycling system. The other is to use the neural network to model the characteristic of each tank, and then serial connection of each neural network that modeled each tank could allow a monitoring of the recycling system. In this study, it was difficult to model the overall characteristic of all tanks by a single neural network because each tank in the treatment system has different role and characteristic. Thus, we used each neural network that modeled the characteristic of each tank, and the overall model of the whole

treatment system was obtained by the connection of each neural network. Fig. 2 showed a proposed modeling protocol for the recycling system. We used principal component analysis (PCA) as a preprocessor of the neural network. Input of the neural network was reduced to 3 principal values from 9 independent variables. The output values of the neural network were COD, total-P (T-P), *o*-P, SS and $NH_4^+$-N in the current tank.

To accomplish a successful modeling, the connectivity within neural networks in the current tank were adjusted to best predict the measured values to be obtained at the next treatment step using SS, $NH_4^+$-N, T-P, *o*-P and COD as input variables.

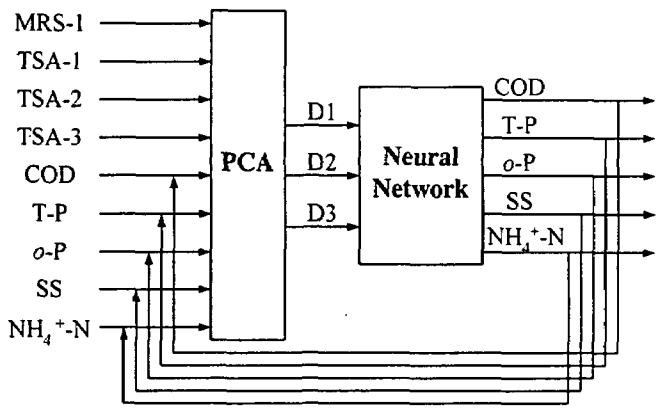


**Fig. 2. A schematic diagram describing training strategy for the neural network in this study. MRS-1, TSA-1, TSA-2 and TSA-3 denote the population density of the bacterial strains. COD (chemical oxygen demand), T-P (total phosphorus), *o*-P (*ortho*-phosphorus), SS (suspended solids) and $NH_4^+$-N are parameters for the wastewater treatment. PCA, D1, D2 and D3 denote principal component analysis and dimensions obtained after the analysis, respectively.**

## 2.5. Ammonium Uptake and Utilization Test

Ability of the isolated heterotrophs to uptake ammonium ($NH_4^+$) was measured to understand an ammonium removal mechanism in the treatment system. The dominant organisms (TSA-1, TSA-2 and TSA-3) were grown in the mineral salts medium [18] containing glucose (0.4% w/v or 3.2% w/v) as a sole carbon source. Unless the organisms were grown on the medium, they were to grown on citrate mineral salts medium [19]. Nitrogen source for these media was $(NH_4)_2SO_4$. The inoculated media were incubated at 26℃ and under rotary shaking (190rpm), and the growth was measured spectrophotometrically (525nm). The ammonium concentrations before inoculation and at stationary phase were measured and the ammonium removal efficiency was calculated.

## 2.6. Extraction of total DNA

Cells used for the ammonium removal test were collected by centrifugation and subjected to total DNA extraction that was performed according to Maniatis et al. [20]. The centrifuged cells were washed once with phosphate buffer and then resuspended in 0.6 ml of lysing buffer (0.15M NaCl, 0.1M EDTA, and 15 mg of lysozyme per ml). After incubation at 37℃for 3 hr, 0.06 ml of 10% sodium dodecyl sulfate was added and the

mixture was incubated at 65℃ for 10 min, and then -70℃ for 5 min. The freeze and thawing procedures were repeated twice. The mixture was then extracted with phenol-chloroform three times and with chloroform once. The alcohol precipitated DNA was resuspended in TE buffer containing RNase A and incubated at 37℃ for 3 hr to remove residual RNA.

### 2.7. PCR Amplification of Glutamine synthetase Gene and Southern Blot Hybridization

Degenerate oligonucleotide primers (forward primer GS-L and reverse primer GS-R) targeting glutamine synthetase genes from the isolated organisms were designed from the conserved GS protein sequences of *Bacillus* sp. including *Bacillus subtilis* 168 (KCTC 1326; ATCC 33234 Spizizen strain 168). The protein sequence alignment and analysis were accomplished using the sequence databases of Gene Bank and the Blast sequence analysis protocol available at National Center for Biotechnology Information (National Institute of Health). Their sequences were 5′-GTG-AAG-TAT-ATC-CGY -CTT-C-3′ (GS-L) and 5′- ATA-YTG-WTC-GCG-YTC-CCA-3′ (GS-R), which were custom-synthesized by GenoTech (Taejon, Korea). One to 3.3 ng of the extracted total DNA were used as a template. Positive control DNA was from *Bacillus subtilis* 168. The PCR procedures for this study were modified based upon the previous report [21] Each PCR reaction mixture (20 $\mu\ell$) contained the following reagents: 10 X *Taq* buffer, MgCl2 (1.5 mM), dNTPs (250 $\mu$M, each), forward primer GS-L (10 pM), reverse primer GS-R (10 pM), *Taq* polymerase (1.25 U) (Promega). PCR was performed in a DNA thermocycler (Perkin Elmer model; GeneAmp PCR System 2400). The PCR conditions were denaturation (94℃, 5min), 30 cycles of the standard PCR (94℃ 1 min; 50℃ 1 min; 72℃ 1 min), and a final chase reaction of (72℃ 5min).

The expected PCR product (1269 bp) from GS gene of *Bacillus subtilis* 168 was identified and nonradioactively labeled using Nonradioactive Labeling and Hybridization Kit (Boehringer Mannheim, Germany). All the following Southern hybridization procedures were done according to the previous report [18] except using CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenylphosphate) (Boehringer Mannheim) as a chemiluminescent substrate for the alkaline phosphatase.

## 3. Results and Discussion

### 3.1. Microbial Identification, and Analyses of the Population Dynamics and Piggery Slurry Treatment

The most dominant heterotrophic bacteria in the treatment system were 4 aerobic bacteria and 3 lactic acid bacteria (LAB). The identified organisms were TSA-1 (*Brevundimonas diminuta*), TSA-2 (*Abiotrophia defectiva*), TSA-3 (*Alcaligenes faecalis*) and MRS-3 (*Streptococcus* sp).

One of the most dominant aerobes was *Alcaligenes faecalis* TSA-3. The most dominant species of LAB was strain MRS-1. Population dynamics of the representative

aerobic bacterium *Alcaligenes faecalis* TSA-3 during the 47-day running period was shown for each tank (Fig. 3). Interestingly, TSA-3 was a predominant species among aerobes in the aeration tank ($10^7 \sim 10^8$ (c.f.u./ml)) but was also observed in the influent and fermentation tanks (Fig. 3). Thus the strain appeared to survive and grow under low oxygen tension and anoxic condition. A reported species of *Alcaligenes faecalis* could oxidize ammonia under aerobic condition and denitrify nitrate ions via NO and $N_2O$ gases under anoxic conditions [22, 23]. *Alcaligenes faecalis* was found to accumulate $NO_2^-$ during exponential growth [19]. Population of the strain MRS-1 was more dominant in the influent and fermentation tanks than aeration and sedimentation tanks, indicating its facultative anaerobic characteristics. The overall population density was in the range of $10^4 \sim 10^7$ (c.f.u./ml).

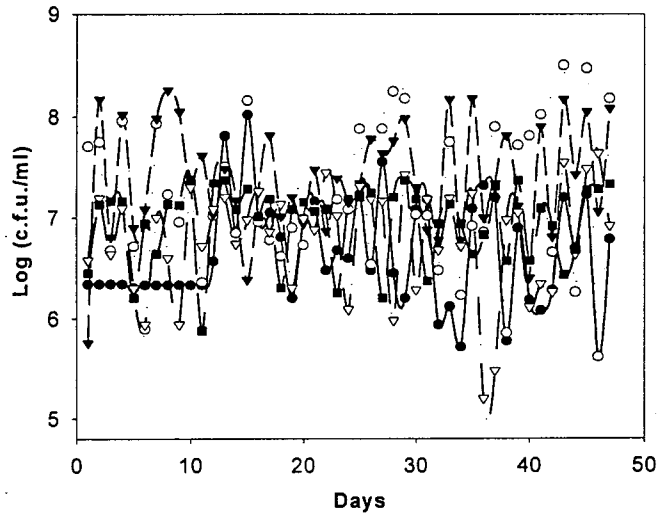


Fig. 3. Population dynamics of a heterotrophic bacterium (*Alcaligenes faecalis* TSA-3) in the recycling treatment system (●- Influent tank; ○- Fermentation tank; ▼- Aeration tank; ▽- Sedimentation tank A; ■- Sedimentation tank D)

The ammonium removal efficiency reached 41% as a maximum. The reason for this rather low efficiency was not clear but unbalanced (presumably, lower) C/N ratio would be one of the causes. Here, however, offensive smells significantly reduced in the effluent.

The overall COD treatment efficiency was about 54%. The COD removal may be mostly accomplished by biological oxidation or absorption (or uptake) of organic compounds derived from livestock feeds that carried abundant carbonaceous, nitrogenous and phosphorus materials, since livestock wastewater contains generally little recalcitrant compounds.

The *ortho* or total phosphorus removal effect was also obvious in the aeration and sedimentation tanks (at least 40%). The possible mechanism for the phosphorus removal would be an uptake of phosphorus by cells under aerobic condition and a subsequent sedimentation of the cells. Surplus phosphorus to be uptaken may be transformed to poly-phosphorus as a storage material within the cells [24]. A discharge of phosphorus

is known to occur under anaerobic conditions [25, 26].

In this study the best removal effect of suspended solids (SS) (63%) was first observed in the aeration tank. This seemed to be due to a transport hole between fermentation and aeration tanks, which screened out most of the sedimented solids.

### 3.2. Principal Component Analysis of Input Data

The input and output dimensions of the neural networks in this study were 9 and 5, respectively. Training data measured for 47 days, was not enough to figure out the complex correlation between the input and output in each tank, and also it was rather hard to expect a generalization. Moreover, there were some noises in the data due to a measuring error or unstable bioprocess. In order to reduce the input and output dimensions, and remove the noisy data, we first used the principal component analysis (PCA) method to analyze the training data. PCA projects high dimensional data onto low dimensional coordinates that consist of principal component axes.

In this study, we used three axes as orthogonal coordinates. These axes were obtained by PCA, removing the data with one-to-many mapping that gives different output from the same inputs. A similar data have been reported previously by Choi et al. [27].

### 3.3. Modeling of Treatment System by Neural Networks

Among 47 training data, we reversed the 6th, 11th, 16th, 21st, 26th, 31st, 36th, 41st, 46th, and 47th data sets for the training phase, which were randomly selected and used as test data to evaluate the generalization performance of the neural network. The neural network has one hidden layer with 30 nodes that were determined by an *ad hoc* method and nonlinear function of each layer except that input layer has a sigmoid. The weight values were adjusted by error back-propagation algorithm.

Through computational experiment we could assure that the learned neural network successfully imitated each tank of treatment system and approximated the target values of the input pattern well. Fig. 4 showed the graphic estimations of COD, $NH_4^+$-N, *o*-P and SS values based upon the neural network analysis. The X-axis represented the tanks from 46th days influent tank to 47th days sedimentation tank 2. As shown in Fig. 4, the proposed neural network could successfully monitor the treatment results according to the population densities of microorganisms. A dramatic increase of the measured SS in the influent at 47th day (Fig. 4 D) was an outlier due to a sampling error.

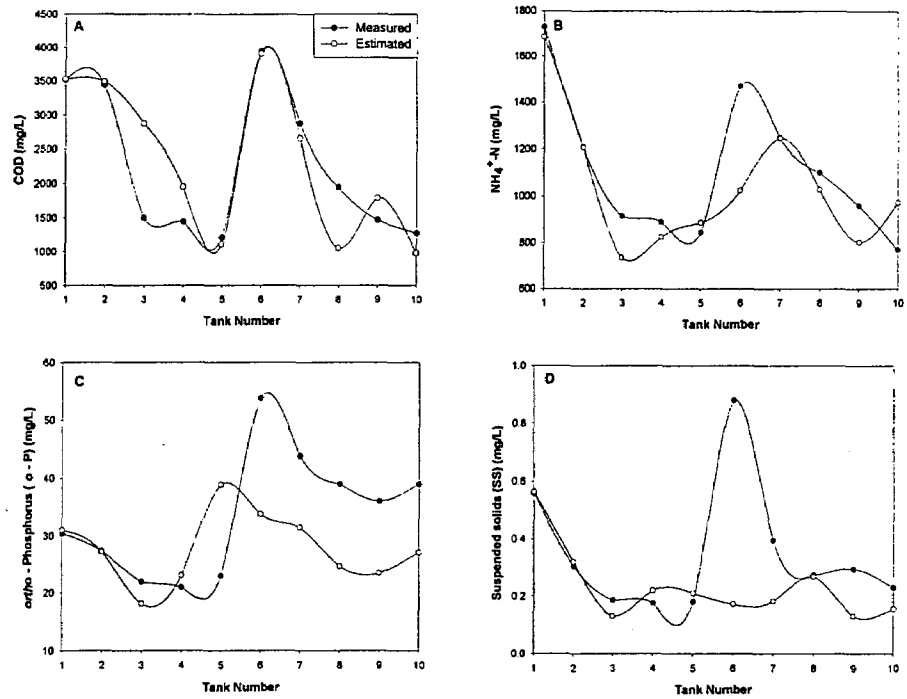


Fig 4. Prediction of various treatment parameters COD (A), $NH_4^+$-N (B), *ortho*-P (C) and SS (D) by the neural network modeling. The serial numbers in X-axis indicate samples taken from tanks of influent, fermentation, aeration, sedimentation-1 and sedimentation-4 at 46 and 47 day's running in order, respectively.

### 3.4. Molecular Analysis of Ammonium Removal

The isolated heterotrophs TSA-1 (*Brevundimonas diminuta*), TSA-3 (*Alcaligenes faecalis*), TSA-4 (not identified) were tested for their ammonium uptake in flask cultures. These three strains could utilize $(NH_4)_2SO_4$ as a sole nitrogen source for their growth. Ammonium appeared to be almost utilized since little ammonium was detected at the stationary phase. Phosphorus removal efficiency was observed up to 60%. $NH_4^+$-N and *ortho*-phosphorus utilization rates appeared to be species or strain specific. This indicates a direct utilization of $NH_4^+$ by a heterotroph and hence removal of nitrogen from the system by circumventing nitrification process that is energy and oxygen consuming pathway. It, therefore, appeared that the ammonium uptake and utilization could contribute to the nitrogen removal in the treatment system (particularly aeration tank).

Amplifications of GS gene with the GS-L and GS-R primers from the isolated heterotrophic bacteria were performed. The GS gene product amplified from *Bacillus subtilis* 168 could hybridize with one of the PCR products from the isolated bacteria

(Fig. 5). Therefore, the presence of ability of ammonium utilization and GS gene in the heterotrophs isolated from the treatment system indicates the possibility that ammonium removal in the system occurs via GS system of these organisms being involved in amino acid synthesis.

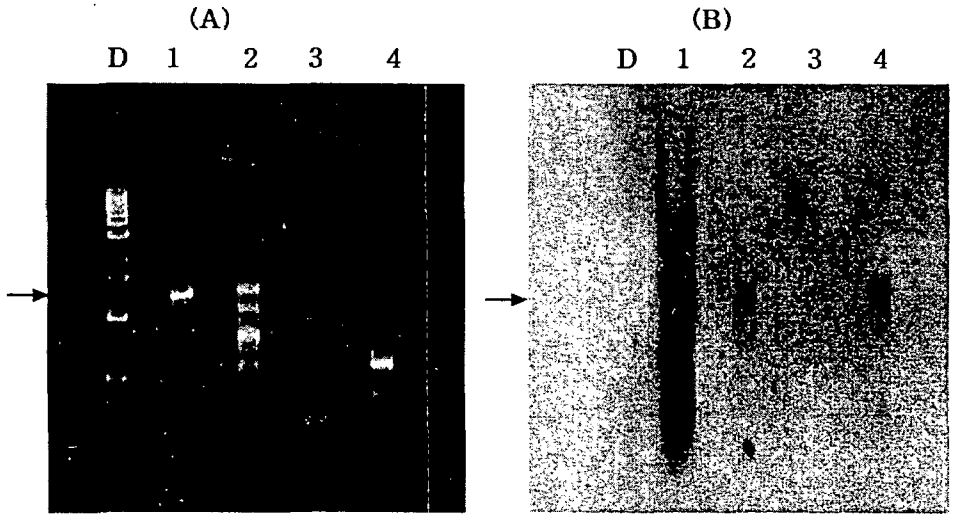


**Fig. 5. Amplification of glutamine synthetase (GS) gene with the GS-L and GS-R primers from the total DNA of heterotrophic bacteria (A) and Southern hybridization with the PCR products using the putative GS gene fragment (1269 bp indicated by arrow heads) of *Bacillus subtilis* 168 (B) as a DNA probe. Hybridization and washing were done under a stringent condition (65℃) . D: DNA 1kb ladder; 1 *Bacillus subtilis* 168; 2 *Alcaligenes faecalis* TSA-3; 3 *Brevundimonas diminuta* TSA-1; 4 TSA-4**

In this paper, we have proposed a novel monitoring system of piggery slurry recycling treatment system. Multi-layer neural networks combined with PCA successfully modeled the tank characteristics. It was possible to train the neural network with the given data by reducing the input dimension with minimal loss of information and removing the noisy data with one-to-many mapping property. The proposed model may be useful to develop a reverse neural network model that could be used to determine optimal microbial densities critical for a desired quality level of the treated wastewater.

The long-term goal of this study will be to construct a real time monitoring system of the recycling treatment for piggery slurry using a multi-layer neural network with an error back propagation learning algorithm. The multi-layer neural network will contribute to modeling a complex relationship between the various population densities of microorganisms and treatment efficiency of the recycling treatment system for piggery slurry and possibly other livestock wastewater.

## Acknowledgements

This work was supported by the Korea Research Foundation (Grant #1998-024-G-00041).

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