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# Immobilizing *Bacillus subtilis* on the carrier of poly (acrylic acid)/sodium bentonite for treating sludge from *Pangasius* fish ponds

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**Abstract:** Sodium bentonite (NaBent) was modified by poly(acrylic acid) (PAAc) to prepare the carriers for immobilization of *Bacillus subtilis*. Different mixtures of NaBent/AAc were regularly dispersed in distilled water and irradiated under gamma rays at an absorbed dose of 6.5 kGy with dose rate of 0.85 kGy/hr in air for polymerization of acrylic acid and formation of poly(acrylic acid)/sodium bentonite (PAAc–NaBent). The reaction yield was determined with the initial concentration of acrylic acid (AAc). The functional group properties of the resulting PAAc–NaBent were analyzed by Fourier Transform Infrared spectra (FTIR). *Bacillus subtilis* cells were immobilized on both NaBent and PAAc–NaBent as carriers by adsorption method for treating the sludge contaminated by fish feces and residual feed from the *Pangasius* farming ponds. The results showed that immobilization capacity of *Bacillus subtilis* on the PAAc–NaBent was better than that on non-modified NaBent. Analysis of BOD for the farming pond water containing *Bacillus subtilis* and the bacteria immobilized carriers with time revealed the lower BOD values obtained with the samples containing PAAc–NaBent, suggested that degradation of organic pollutants by *Bacillus subtilis* immobilized on the PAAc–NaBent was faster than that by free bacteria.

**Keywords:** *Gamma irradiation, bentonite, acrylic acid, Bacillus subtilis*

## I. INTRODUCTION

Bentonite (Bent) is a kind of pure clay consisting mostly Montmorillonite (MMT) with the lamellar structure. To be a mechanically, chemically and thermally stable material, it was widely studied and applied in many fields from industry, agriculture to domestic and pharmaceutical chemistry [1]. For example, polyacrylate modified Bent was used as the heat-resistant and solvent-resistant materials [2, 3, 4]. Another example is that Bent and MMT were modified as absorbents for removal of heavy metal ions and dyes from industrial wastewater [5-7, 12]. Similar to other inorganic materials, Bent was studied as the carriers for immobilization of microbial cells. This is a very effective and promising carrier due to its advanced properties such as durable structure, modifiable bio-activity and multiple times for reusing [8].

*Bacillus subtilis*, a bacterial strain that commonly found in the soil and the gastrointestinal tracts of ruminants and human, can synthesize different enzymes such as amylase, protease, lipase… facilitating biodegradation of organic compounds, which contaminate the wastewater. Recently, these bacteria were immobilized on Amberlite XAD-4, an inorganic material based on MMT, and used as a new biosorbent in trace metal determination [9]. Enzymes such as β-amylase and alkaline protease manufactured by *Bacillus subtilis* were also immobilized on the hydrogel carriers for further applications [10-12].

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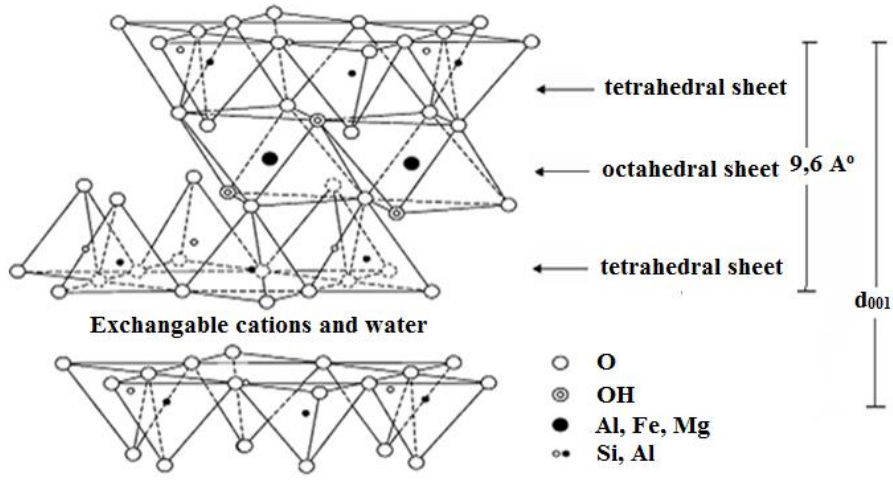


**Fig.1.** Structure 2:1 of MMT [1]

In the present study, PAAc were polymerized in the lamellar spaces of Sodium Bentonite (NaBent) by gamma irradiation. Resulting poly (acrylic acid)/sodium bentonite (PAAc-NaBent) with increased number of active groups has been used as the carrier for immobilization of *Bacillus subtilis*. Biochemical oxygen demand (BOD) of the contaminated sludge from the *Pangasius* farming ponds was investigated in the presence of *Bacillus subtilis* or the bacteria immobilized on the PAAc-NaBent for evaluation the usage of this carrier in practice.

## II. EXPERIMENTAL

### Material

Acrylic acid was purchased from Merck, Germany. Trypticase Soya Broth (TSB) medium for bacterial incubation was provided from Himedia, India. Sodium bentonite mineral was extracted in Binh Thuan province, Vietnam. *Bacillus subtilis* bacterium with activity of $9 \times 10^9$ CFU/g was supplied from Nanogen Biopharmaceutical Co., Vietnam. Distilled water was used in all experiments.

### Preparation of PAAc-NaBent material

Sodium bentonite powder (5g) was added in 10 ml aqueous solution of 10 % (w/v) AAc for 4 hrs. The mixture was irradiated under Cobalt – 60 gamma ray at the dose rate of 0.85 kGy/hr. The same experiments were also performed with the aqueous solutions of 17.5 % and 25.0 % AAc.

### Determination of characteristic properties

Polymerization yield is the ratio of the initial AAc monomer converted into PAAc polymer after extracting the irradiated product in distilled water. The reaction yield (PY) was calculated as following formula [13]:

$$PY\ (\%) = \frac{m_1 - m_2}{m_0} \times 100 \qquad (1)$$

Where $m_0$ is the initial weight of AAc monomer, $m_1$ is the dried weight of the irradiated sample after extracting in distilled water, and $m_2$ is the initial weight of NaBent.

Fourier transform infrared (FT-IR) spectroscopy was used to investigate the gamma radiation effects on the chemical structure of PAAc/NaBent. The samples were prepared by KBr pellet method, and their FT-IR were recorded by spectrophotometer (8400S, Shimadzu, Japan) in the wave number between 500 and 4000 $cm^{-1}$ at scanning number of 20.

**Immobilization of Bacillus subtilis bacteria on PAAc-NaBent carrier**

NaBent and PAAc-NaBent obtained by radiation modification of NaBent with of 17.5 % AAc were studied as carriers for immobilization of *Bacillus subtilis*. Each carrier (2g) was dispersed in a flask containing 10 ml suspension of *Bacillus subtilis* with cell density of $9\times10^9$ CFU/ml at pH ~ 7.0. The mixture was incubated at 37 $^oC$. After 24 hrs incubation, the *Bacillus subtilis* immobilized carrier was separated using a centrifuge (ABE 12, Hettich) at 4000 rpm for 20 min., then dried at room temperature and stored at 4 $^oC$ for further experiments.

After a certain period of time, the immobilized carrier was taken out, and number of *Bacillus subtilis* cells remained in the residual suspension was determined in order to investigate the immobilization efficiency of the bacteria on carrier (H) [14-16]. Briefly, the remained suspension was diluted to a dilution factor of $10^{-6}$ by using a ten-fold serial dilution procedure in the sterilized saline solution (0.9 % NaCl). These serial dilutions were spread on the agar plates containing the 3 % TSB (w/v) medium, and then the plates were incubated in control incubator at 37 $^oC$ for 48 hrs. The total number of viable colonies (total plate count method), and the immobilization efficiency was calculated as follows:

$$\text{E (cfu/g)} = \frac{\sum C}{d[(n_1v_1 + n_2v_2(0.1)]} \quad (2)$$

Where Σc is the total counted colonies (with condition having enough 25-250 colonies per plate); $n_1$ and $n_2$ are the colony numbers of plates in the first and second dilution, respectively; $v_1$ and $v_2$ are the volumes of spreading suspensions corresponding to the first and second dilutions; d is the dilution from which the first counts were obtained.

$$\text{H (\%)} = \frac{E_0 - E}{E_0} \times 100 \quad (3)$$

Where $E_0$ and $E$ are the total cell numbers (CFU/g) in the initial and residual suspensions of *Bacillus subtilis* after immobilization, respectively.

**Release of Bacillus subtilis from the modified carrier into aqueous solution**

1 g of the dried *Bacillus subtilis* immobilized carrier was added into a flask containing 20 ml saline solution and lightly shaken for 15 min, then the solution was centrifuged at 4000 rpm for 20 min for separation of the carrier. The number of *Bacillus subtilis* cells released from the carrier was determined from supernatant. This centrifugation process was repetitive over 35 times at which the number of living cells released to the saline solution was very small.

**Test of Biochemical Oxygen Demand (BOD)**

Biochemical oxygen demand (BOD) was the amount of dissolved oxygen (DO) demanded by aerobic biological organisms to break down organic material (sludge) present in a given liquid sample at a certain temperature over a specific time period. The lower the BOD, the higher the degradation capacity of bacteria.

Free *Bacillus subtilis* and the bacteria immobilized PAAc-NaBent were separately dispersed in TSB medium to prepare the suspensions with cell density of $2 \times 10^5$ CFU/ml. 10 mL bacterial suspension was mixed in 1 litre of the diluted sludge from *Pangsius* fish pond for sampling. Number of cells in these suspensions was determined by the total plate count method without removing the sludge. The dissolved oxygen (DO) of these samples (the water contaminated with the slude containing free bacteria or carrier) were

determined before and after incubation using the SMEWW 5210 B:2005 standard method in Quality Assurance and Testing Center 3 [13] and their BOD were calculated using following formula [18]:

$$\text{BOD (mg/L)} = \frac{D_1 - D_2}{P} \quad (4)$$

Where $D_1$ and $D_2$ are the initial and final DO of the sludge sample after incubated with the free bacteria or the bacteria immobilized on carrier in various period of time and $P$ is decimal volumetric fraction of the samples of water contaminated with the sludge.

## III. RESULT AND DISCUSSION

### Efficiency of polymerization

Because of polarity of funtional groups (carboxyl groups), AAc monomer had a tendency to come in the space between lamellars where cations stayed. By gamma irradiation, the PAAc polymers were synthesized among these lamellars (Fig. 2).

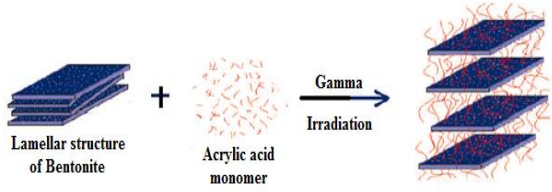


**Figure 2.** Polymerization process of AAc on Bentonite carrier

Fig. 3 showed the effect of absorbed dose on the polymerization yield (PY) of AAc in the mixture sollutions of the same NaBent amount and various AAc contents. It is obviously that the yield of polymerizxation reaction steadily increased with the radiation dose and AAc content in the mixture. At the dose of 6.5 kGy, the PY values obtained from the solutions of 10 % and 17.5 % AAc were the same, but it increased to 20% with the solution contains 25% AAc. The results also suggested that AAc continuously polymerized by increasing of absorbed dose, and the highest PY was about 23% obtained from the mixture of NaBent and 25% AAc irradiated at 25 kGy.

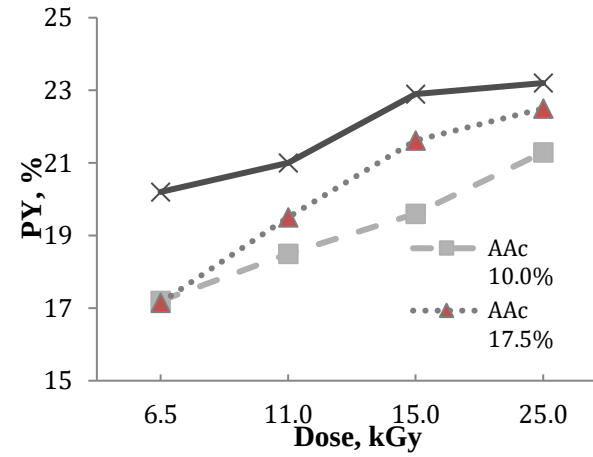


**Figure 3.** Effect of absorbed doses on the percentage yield of PAAc polymerization

### FT – IR spectra

FT – IR had been used to determine carboxyl group (-COOH) of PAAc formed by radiation polymerization. Peak at wave number between 1710 and 1730 $cm^{-1}$ represented for the characteristic absorption frequency of C=O in carboxyl groups. In addition, another peak which was assigned to the bending vibrations of O-H bonds in $Al_2$-OH in octahedral sheets of NaBent appeared at 915 $cm^{-1}$ [19].

Result in the Fig. 4 illustrated that there was a change in the structure of PAAc/NaBent by gamma irradiation. The characteristic peak of carbonyl group observed at 1726 $cm^{-1}$ revealed the presence of PAAc in the modified NaBent. It was stronger for the PAAc/NaBent obtained from solution containing 25% AAc, and became weaker for the PAAc/NaBent from lower AAc concentrations of 10% and 17.5%.

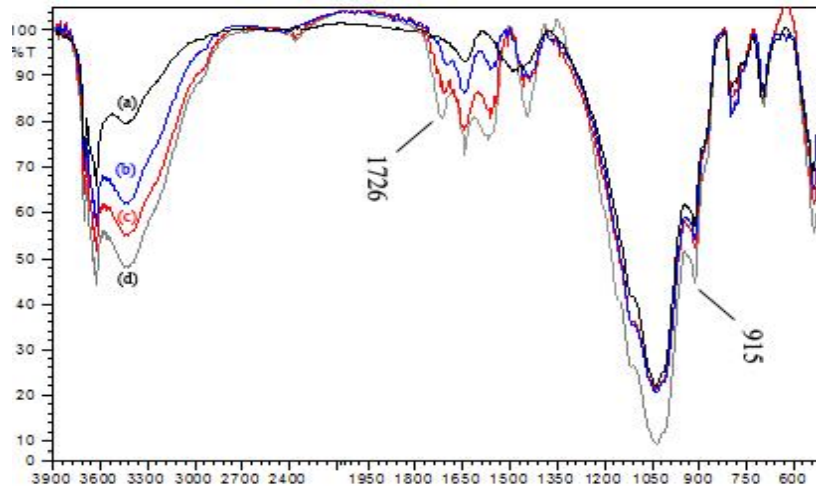


**Fig. 4.** IR spectra of the initial (a) and modified NaBent at various AAc concentrations: 10% (b), 17.5% (c) and 25% (d) at 6.5 kGy

**Immobilization of Bacillus subtilis on the carrier**

There are some methods for immobilizing microbial cells on the inorganic carriers. Binding of cell to a carrier by physical adsorption is easy way of immobilization. This method was based on the physical interaction between the microorganism and carrier surface. In this experiment, *Bacillus subtilis* cells were immobilized on both NaBent and modified NaBent carriers by adsorption via formation of hydrogen bonds between bacteria and carboxyl groups of the carrier. Positive charged hydrogen of the structural protein in the bacteria membrane may bind to the negative charged oxygen of carbonyl group in the carrier as follows:

$$\delta^- N - H\ \delta^+ \qquad (5)$$

$$\delta^- O = C\ \delta^+ \qquad (6)$$

$$\delta^- N - H\ \delta^+ \cdots\cdots \delta^- O = C\ \delta^+ \qquad (7)$$

After immobilization, number of *Bacillus subtilis* remained in the residual suspension and immobilization efficiency were determined and presented in the Table I. Depends on the adsorption capacity of the carrier, the decrease of remained cells was. The more bacteria were immobilized on the carrier, the lower number of cells remained on the residual suspension was. As one can see from the Table, number of cells remained in the bacteria suspension after immobilization period by NaBent was higher than that by the modified NaBent, suggested that the immobilization efficiency of *Bacillus subtilis* on the PAAc-NaBent was 99.9 %, much better than that on initial NaBent of 97.5 %.

**Table I.** Number of colonies of *Bacillus subtilis* suspension after immobilized on various carriers and their efficiency of immobilization

| **Carrier** | **Number of cells, E (CFU/ml)** | **H (%)** |
|---|---|---|
| NaBent | $3.5 \times 10^7$ | 97.5 |
| PAAc-NaBent | $4.1 \times 10^5$ | 99.9 |

**Release of Bacillus subtilis from modified carrier into aqueous solution**

Release of bacteria to aqueous solution during centrifugation is an important property to estimate the reuse capacity of the immobilization materials. The slower the release of bacteria is, the higher the reuse cycle of immobilization material is.

Therefore, the release of microbial cells from the carriers should be controlled. After certain cycles of centrifugation, the bacteria number released from the carrier into the aqueous solution was determined and the results were presented in the Table II. As one can be observed, the numbers of released cells were significantly reduced by increasing of centrifugation cycles from 5 to 35.

**Table II.** Number of *Bacillus subtilis* cells released from the carriers into aqueous solution after various cycles of centrifugation

| **Number of centrifugation cycle** | **Number of cells (CFU/ml)** | |
|---|---|---|
| | **PAAc-NaBent** | **NaBent** |
| 5 | $3.64\times10^4$ | $5.25\times10^6$ |
| 15 | $7.15\times10^3$ | $3.21\times10^4$ |
| 25 | $5.85\times10^2$ | $7.53\times10^3$ |
| 35 | $2.15\times10^1$ | $1.95\times10^2$ |

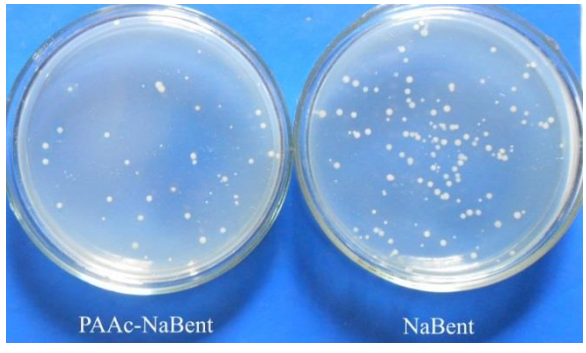


**Fig. 5.** Number of *Bacillus subtilis* released from PAAc-NaBent and NaBent into aqueous solutionafter 5 cycles of centrifugation (dilution at $10^{-4}$)

It was obviously that the number of bacterial cells released from the PAAc/NaBent carrier was smaller than that from NaBent. For example, it was $3.64\times10^4$ CFU/ml for the PAAc/Bent compared with $5.25\times10^6$ CFU/ml for NaBent after 5 cycles of centrifugation. Thus, the release of bacteria from the PAAc/NaBent was slower than that from the initial NaBent, namely immobilization efficiency of *Bacillus subtilis* on the PAAc modified NaBent was better than that on non-modified NaBent.

**BOD test**

Table III shows the BOD values of diluted sludge with the presence of *Bacillus subtilis* in suspension and the bacteria immobilized on PAAc/NaBent carrier with the same cell density of $2\times10^5$ CFU/ml over incubation time. After 5 days incubation at 20ºC, BOD of both sollutions containing the same amount of sludge from *Pangasius* fish pond treated with free *Bacillus subtilis* and the bacteria immobilized PAAc/NaBent decreased to 4.5 and 4.4 mg/L, respectively. Thus, there is no significant difference in the degradation capacity of *Bacillus subtilis* in suspension and on the immobilization material. However, this value of the sludge solution after 10 day incubated with free bacteria was higher than that with immobilized bacteria, namely that the degradation of sludge by the immobilized bacteria was better than that by free bacteria. It may be due to the bacteria aggregated to emulsion state in solution. After long enough time of incubation, the aggregation prevent sludge contact to the bacteria, reduced their degradation rate, especially in the bottom, while the sludge can easily entrap to the carrier, and be degraded by *Bacillus subtilis*.

**Table III.** BOD values (mg/L) versus the incubated period of 3 days ($BOD_3$); 5 days ($BOD_5$) and 10 days ($BOD_{10}$)

| **Sample** | **$BOD_3$(mg/L)** | **$BOD_5$(mg/L)** | **$BOD_{10}$(mg/L)** |
|---|---|---|---|
| Free *Bacillus subtilis* | 7,6 | 4,5 | 2,5 |
| Immobilized *Bacillus subtilis* | 7,8 | 4,4 | 1,2 |

## IV. CONCLUSION

PAAc modified NaBent have been prepared by gamma irradiation method at various radiation doses range from 6.5 to 25 kGy. The IR spectra proved that carbonyl group formed in the modified NaBent. The immobilization efficiency of *Bacillus subtilis* on the PAAc/NaBent was better than that on initial NaBent, while release of bacteria from the immobilization material of PAAc/NaBent carrier was slower than that of NaBent. Degradation rate of sludge gathered from the *Pangasius* fish pond by the *Bacillus subtilis* immobilized on the PAAc/NaBent carrier was higher than that by the free bacteria after 10 day incubation at 20°C, suggested that this immobilization material may be applied to reduce the contamination for the *Pangasius* farming ponds.

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