Book Chapter

Isolation of Flocculant-Producing Bacteria in Wastewater and its Application for Wastewater Treatment in the Mekong Delta, Vietnam

Cao Ngoc Diep^{1*}, Bui The Vinh² and Huynh Van Tien³

¹Biotechnology R&D institute, Can Tho University, Vietnam

²CanTho Dairy factory, Vietnam

³KienGiang University, Vietnam

***Corresponding Author:** Cao Ngoc Diep, Biotechnology R&D institute, Can Tho University, Vietnam

Published **August 02, 2021**

How to cite this book chapter: Cao Ngoc Diep, Bui The Vinh, Huynh Van Tien. Isolation of Flocculant-Producing Bacteria in Wastewater and its Application for Wastewater Treatment in the Mekong Delta, Vietnam. In: Malik Badshah, editor. Prime Archives in Biosciences. Hyderabad, India: Vide Leaf. 2021.

© The Author(s) 2021. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License(<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Summary

The discharge of poorly treated effluents into the surface water such as streams, rivers and lakes had far – reaching impacts on human activities and aquatic life forms. Therefore, it needs to be cleaned to acceptable levels. For polluted water and wastewater treatments, biological methods are often preferred; in which the usage of highly efficient bioflocculants produced by flocculants

producing bacteria (FPB) were selected because low cost, harmlessness to human and environment. In order to reduce water pollution in fish ponds and treat wastewater piggery of post-biogas in pig farms in the Mekong Delta, Vietnam, Protein FPB isolates and Polysaccharide FPB isolates were isolated from soil and water samples; identified by 16S rRNA gene sequencing; and then investigated its characteristics and flocculation activities. The good isolates were selected to optimize parameters of flocculation and its applications in treatments of the polluted water of tra-fish ponds and the wastewater piggery of post-biogas in pig farms. Targets of the quality of output wastewater were compared to standards of the Regulation QCVN40 (A standard (mg/L): TSS < 50; COD < 75; BOD₅ < 30; TN < 20; Nitrite (NO₂⁻) < 0.01; Nitrate (NO₃⁻) < 50; TP < 4 and PO₄³⁻ < 6).

From the polluted water in tra-fish ponds: From 155 samples of 10 sites in the Mekong Delta, 389 FPB isolates were isolated including 154 Protein FPB isolates and 235 Polysaccharide FPB isolates. Among the FPB isolates, a total of the 20 isolates (10 of Protein FPB isolates and 10 of Polysaccharide FPB isolates) having the highest flocculating efficiency were selected to identify, the results showed 16 strains belonged to genus *Bacillus*, 2 strains belonged to genus *Staphylococcus*, 1 strain was genus *Arthrobacter* and 1 strain was *Agrobacterium*, respectively. Among the 20 isolates, the polysaccharide FPB *Agrobacterium tumefaciens* strain STT37PS (optimal growth medium: adding 1% sucrose and 5% glutamic) was selected to treat wastewater of three-month old bronze featherback (ThacLac) fish and ababas (RoDong) fish ponds. The quality of treated wastewater reached the standards of Regulation QCVN40 in a short time (3 days for bio-floc stage, 2 days for *Lemna* stage).

From the wastewater piggery of post-biogas in pig farms: One hundred-nineteen (119) isolates including Protein FPB isolates (53.85 %) and 102 Polysaccharide FPB (46.15 %) isolates were isolated from 147 samples collected from 13 cities and provinces in the Mekong Delta. A total of the 34 isolates (18 of Protein FPB isolates and 16 of Polysaccharide FPB isolates) having

the highest flocculating efficiency were chosen to identify. Among of them, all of the 18 Protein FPB isolates belonged to genus *Bacillus*; the 16 Polysaccharide FPB isolates belonged to genus *Bacillus* (majority) (10/16), genus *Klebsiella* (3/16), *Ochrobactrum anthropi* (2/16) and genus *Sphingobacterium* (1/16), respectively. Out of the 34 isolates, 2 strains had the highest flocculating activities were *Bacillus megaterium* LA51P (Protein FPB) and *Bacillus aryabhattai* KG12S (Polysaccharide FPB). Combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S applied in the wastewater treatment (at volume: 1 m³ and 50 m³) showed that the criteria of the treated wastewater were lower than the standard A of the Regulation QCVN40.

Keywords

Bacillus; Flocculation; Flocculant-Producing Bacteria; Polluted Water in Tra-Fish Ponds; Wastewater Piggery

Introduction

Most of the water people use everyday comes from the sources of surface water such as streams, rivers and lakes. Humans tend to have clean water but at the same time, the rate of pollution in water was increased by their activities. Many lakes, rivers and streams are becoming polluted increasingly by industrial and agricultural activities, and domestic wastes are often discharged into the surface water without proper treatment especially. Therefore, it is necessary to treat before reuse by evaluation of the physical, chemical and biological characteristics of the water in order to verify whether the observed water quality is suitable for the intended uses [1]. It has been suggested that it is the leading worldwide cause of deaths and diseases and accounts for the deaths of more than 14000 people daily.

The flocculation is a preferred method because of its high efficiency and simplicity [2]. Flocculants can be classified into three groups: inorganic flocculants (polyaluminum chloride and aluminum sulfate), organic synthetic flocculants (polyethylene

imine and polyacrylamide derivatives), and naturally occurring flocculants (chitosan, tannins, and bioflocculants) ([3-5] Roselet, et al., 2015). Currently, many chemical flocculants including polyaluminum chloride, aluminum sulfate, and polyacrylamide are being applied in industrial processes such as wastewater treatment, downstream processing of biopharmaceutical proteins, dehydration of activated sludge, tap water production, dredging and fermentation. However, the extensive use of chemical flocculants has been restricted because of their neurotoxic and carcinogenic properties [6]. With the restriction of the use of chemical flocculants, it is necessary to use of the alternative flocculation technology.

Bioflocculation is a dynamic process resulting from the synthesis of extracellular polymer by living cells.

When compared with conventional synthetic flocculants, bioflocculant has more special advantages in their use such as safety, strong effect, biodegradable and harmlessness to humans and the environment, so they may potentially be applied in drinking and wastewater treatment, downstream processing, and fermentation processes [3].

As an alternative to chemical flocculants, bioflocculants have been widely studied in recent years as a promising option for wastewater treatment because of their improved efficiency, innocuity and biodegradability compared with that of traditional flocculants [7-12]. Since 1984, when Fattom and Shilo found that *Phormidium* J-1 could produce a polymer to flocculate bentonite [13], many microorganisms have been studied for their ability to produce bioflocculants. For example, *Bacillus* sp. AEMREG7 was shown to produce a bioflocculant with a maximum flocculating activity of 92.6% against kaolin clay suspension [14]; *Enterobacter cloacae* sp. WD7 and *Pseudomonas alcaligenes* WD22 were shown to produce bioflocculants with flocculating activities of 91% and 55%, respectively [15]. Fungi such as *Aspergillus parasiticus* was able to produce a bioflocculant with a flocculating activity of 92.4% against Reactive Blue 4 and a bioflocculant produced from *Aspergillus niger* had a flocculating activity of 63% for turbidity

removal [16,17]. *Actinomyces* such as *Nocardia* spp. were able to produce a highly efficient bioflocculant [18]. Algae such as *Desmodesmus* sp. F51 were found to produce a bioflocculant (Poly- γ -glutamic acid) with a flocculating activity of 96% [19]. Consequently, the screening of new strains that produced highly efficient bioflocculants with low cost production, little impact on the environment became an important research topic in this field [4,20]. In addition, the applications of bioflocculants in the control of harmful algae blooms in the water environment has great practical significance.

In this study, flocculants producing bacterial isolates were isolated, identified from water and soil in the Mekong Delta and then investigated its characteristics and flocculation activity. Response surface methodology (RSM) was used to optimize the parameters of the bioflocculation for treating by kaolin suspension. The results of the study suggested that these bioflocculants offered a highly efficiency, it may be supplied the solution for the wastewater treatment and applications in the sustainable agricultural production further.

Materials and Methods

Materials

Soils and water samples from tra-fish (catfish) pond, shrimp pond and piggery wastewater were collected in the sterile plastic bags or sterile plastic bottles 1-L (sterilized by alcohol 70%). The samples were transferred or stored in ice-box before moving to the laboratory (Can Tho University) and stored in the refrigerators (-5°C) until isolation.

Methods

Isolation of Flocculant-Producing Bacteria

Following ten - fold serial dilution, an aliquot of 100 μ L of the sample was spread over the cultivation agar media for the isolation of Protein FPB [21] and Polysaccharide FPB [22]. The cultivation agar plates were incubated at 30 °C overnight.

The subcultures were incubated under aerobic conditions at 32 °C for 24 h. This bacterial strain was consistently cultivated on nutrient agar and preserved in the glycerol solution (20% w/v) and stored at -80 °C.

Composition of the Used Media

Two types of media, namely, seed media and production media, were used to screen and obtain flocculants producing bacteria. The seed medium had the following composition (in g/L): 20 g of glucose, 0.5 g of yeast extract, 50 g of glutamate, and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH was adjusted to 7.0 - 0.2. [21]. The production medium contained the following components (in g/L): 10 g of glucose, 0.5 g of yeast extract, 1.5 g of urea, 5 g of KH_2PO_4 , 0.1 g of K_2HPO_4 , 0.1 g of NaCl, and 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH was adjusted to 7.0 - 0.2, 0.3 g of carbamate [23].

Determination of Flocculating Activity

Flocculation of Kaolin:

Flocculation dynamics of cells and bioflocculant-producing was measured according to the used kaolin suspension method (Figure 1 and 2).

Figure 2.1: Flocculation mechanism of the Kaolin suspension [23].

Figure 2.2: Flocculation mechanism of the bioflocculant DYU500 [24].

One mL agent was mixed into 50 ml of 4 g/ L the kaolin suspension in a 50 ml graduated cylinder covered with clean film. The test cylinder was gently shaken and settled at room temperature. At prescribed time, 3 mL of supernatant was carefully removed from the upper layer of solution and measured the absorbance at a 550 nm. Control experiment was also conducted without flocculation agent.

Using a suspension of kaolin clay as test material, flocculating activity was determined according to Kurane *et al.* [25] as modified by Gao *et al.* [26]. A suspension of kaolin clay (4 g/L) in deionized water at pH 7 was used as a stock solution for the subsequent assays. The following solutions were mixed in a test tube: kaolin clay suspension (9 mL), culture supernatant (0.1 mL) and 1% CaCl_2 (0.25 mL). A control in which the culture supernatant was replaced with deionized water was also included and measured under similar conditions. The final volume of all mixtures was made up to 10 mL with deionized water. The solutions were mixed gently and allowed to settle for 5 min at room temperature. The optical density (OD) of the clarifying upper phase solution was measured at 550 nm using a Thermo Spectronic spectrophotometer (Helios Epsilon, USA) and the flocculating activity determined as follows: Flocculating activity = $[(B - A)/B] \times 100\%$ where A and B are optical densities at 550 nm of the sample and control, respectively.

The flocculating activity was calculated using the following expression [27]:

$$\text{Flocculating activity \%} = (A_c - B_s)/A_c \times 100 \quad [28] \quad (1)$$

where A_c and B_s represent the OD of the control and real samples, respectively.

Optimization of Cultural Conditions for Biofloculants Production:

a. Effects of pH

The effect of pH on the production medium was determined at different pH values ranging from 3 – 11 and adjusted by adding 1N HCl and 1N NaOH as needed. A fresh culture of 2% (v/v) bacterial isolate was inoculated into the prepared medium, incubated for 72 h at 35°C, and shaken at 150 rpm. The flocculating activity was examined using the kaolin clay to check the optimal pH required for biofloculant production as indicated above [29].

b. Effects of Inoculum Sizes

The influence of the inoculum volume on biofloculant production by bacterial isolate was examined because different inoculum sizes exert certain effects on the flocculation activity and cell mass growth. The inoculum sizes used were 0.1%, 0.5%, 1%, 2%, 5%, and 10% [29].

c. Effects of Temperature and Shaking Speed

The bacterial isolate was inoculated into the seed media and incubated at 37 °C on a shaker at 150 rpm for 24 h. From the fresh culture of 2% v/v, was inoculated into several sets of 200 mL bottles containing 50 mL of the production medium, then incubated at different cultivation temperatures (25, 30, 35, 40 and 45°C) on shaking 150 rpm for 144 h. Also, the shaking speeds were investigated for different speeds such as 100, 120, 140, 160, 180, 200 and 220 rpm, respectively. The cell-free supernatant was obtained by a centrifugation of 4000 rpm for 30 min to separate the cells. Then the flocculating activity was checked.

4. Effects of Carbon and Nitrogen Sources

Biofloculants production by microorganisms is significantly influenced by carbon and nitrogen sources [4]. These parameters were assessed according to Lachhwani [30]. The growth media were prepared in separate flasks. The bacterial strain was inoculated into the prepared medium. The media were supplemented with 1 g/L each of various carbon sources, incubated at 30 °C, and shaken at 150 rpm for 7 days. To determine the influence of nitrogen sources on biofloculants production, 0.5 – 5 g/L each of the various nitrogen sources was integrated into the fermentation medium in separate containers, and the flocculation activity was calculated according to Lachhwani using Equation (1) [30].

Identification of FPB

The bacterial strain was identified using molecular technique based on the 16S rRNA gene amplification by polymerase chain reaction (PCR) followed by sequencing of the amplified gene as designed according to Neumann et al. [31] and Jie et al. [32].

Table 2.1: The effects of nutritional factors on biofloculants production

Carbon sources	Nitrogen sources	Mineral sources
Glucose (1%)	Urea (5%)	KCl (0.3%)
Sucrose (1%)	Yeast extract (0.05%)	FeCl ₃ (0.5%)
Starch (1%)	NH ₄ (SO ₄) ₂ (0.05%)	CaCl ₂ (0.5%)
	Glutamate (0.05%)	K ₂ HPO ₄ (0.2%) + KH ₂ PO ₄ (0.5%)

Cells were incubated in 250 mL flasks containing 50 mL fresh Luria Bertani broth (LB) for 16 h at 37°C with shaking (120 rpm). The genomic DNA of the strain was then extracted using CTAB method of DNA extraction from microorganisms. PCR amplification was carried out to determine the partial 16S rRNA gene. The PCR program was 30 cycles of 94°C (1 min), 55°C (30 s), and 72°C (1.5 min) [3]. The PCR primers were 37F 5-CCAGCAGCCGCGGTAATACG-3 (forward) and 1497R 5-TACCAGGGTATCTAATCC-3 (reverse). Purification of the PCR products and the determination of sequences were performed by TBR Company, HCMC. The 16S rRNA gene

sequence of the strain obtained was compared with the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Wastewater treatment with Biofloculants

Application of FPB to Remove Ammonia in Tra-Fish (Catfish) Ponds

- Experiment in vitro.
- Experiment in ponds.

Application of FPB to remove Ammonia in Piggery Wastewater

- Experiment in vitro.
- Experiment in containers: 10-L, 100-L and pond (50 m³).

Results and Discussions

The agricultural characteristics in the Mekong Delta are rice cultivation, horticulture, aquaculture (especially tra-fish [*Pangasius*] farms), husbandry as mainly raise pig at family scales and few of pig farms (1000 – 5000 pigs/farm). Therefore, application of FPB in wastewater treatment of tra-fish farming, raise pig were presented.

Applications of FPB in Water Treatment of the Tra-Fish Farming

From 155 samples, 389 FPB isolates were isolated including 154 protein FPB isolates and 235 polysaccharide FPB isolates. Among of them, 304/389 isolates had the flocculating activities more than 50% including 117 protein FPB isolates and 187 polysaccharide FPB isolates.

In the Mekong Delta, 10 cities/provinces had suitable conditions to develop tra-fish farms. The water/sludge samples were collected from these tra-fish farms. The results of the isolation and the flocculating activities of the bacterial isolates were presented in Table 3.1.

Table 3.1: FPB isolates isolated from the 10 sites in the Mekong Delta.

No	Site	No of sample	Number of isolates isolated			Isolates had the FA >50%		
			Total	Protein	Polysaccharide	Total	Protein	Polysaccharide
1	AnGiang	24	19	10	9	14	7	7
2	BenTre	14	56	26	30	56	26	30
3	CanTho	21	33	10	23	31	10	21
4	DongThap	21	73	24	49	35	10	25
5	HauGiang	15	23	6	17	19	6	13
6	KienGiang	11	50	23	27	49	22	27
7	SocTrang	12	49	19	30	23	9	14
8	TienGiang	08	18	9	9	18	9	9
9	TraVinh	12	45	18	27	36	9	27
10	VinhLong	17	23	9	14	23	9	14
	Total	155	389	154	235	304	117	187

Note: FA: flocculant activity

All of the FPB isolates isolated were amplified with 37F and 1479R primers. After PCR, bands at 1500 bp position were identified, therefore they were the FPB strains.

The FPB strains developed very well on the media from 48 - 72 h at 30°C. The colonies had round-shape, spreading, slimy, smooth, transparency, colorless or milk-color, white or yellow. Most of the colony size of the strains were small (1 – 1.5 mm in diameter), while a few strains were large (2.5 – 3 mm in diameter) (Figure 3.1).

Morphological characteristics of the FPB cells were short rod-shaped and circular. The cell had an outer mucus and viscous layer that helped to flocculate the organic and inorganic suspended particles and settle at the bottom of containers. (Figure 3.2)

Figure 3.1: Shapes, sizes and color of a few FPB colonies isolated

Figure 3.2: Shapes of several FPB cells isolated.

Identification of the FPB Isolates

Among the FPB isolates, a total of the 20 isolates (10 of Protein FPB isolates and 10 of Polysaccharide FPB isolates) with the highest flocculating efficiency were selected to identify by 16S rRNA genes sequencing.

The results of the identification in table 3.2 and 3.3 showed that among the 20 isolates, the 16, 2, 1 and 1 isolates belonged to genus *Bacillus*, *Staphylococcus*, *Arthrobacter* and *Agrobacterium* respectively.

The Protein FPB Isolates:

The result in figure 3.3 showed that the phylogenetic tree had 2 clusters: Cluster A had 2 small clusters as cluster A1 with two strains: *Bacillus aryabhattai* DTT07P and *Staphylococcus xylosus* TVT05P having high similarity with *Bacillus amyloliquefaciens* TGT03P

Table 3.2: Phylogenetic affiliation of 10 protein FPB isolates on the basis of 16S rDNA gene sequences by using BLAST programme in the GenBank database based on sequence similarity

No	Strain	Number of nucleotids	Closest species relative	Similarity (%)
01	AGT08P	1151	JQ229803 <i>Bacillus megaterium</i> strain 1CK24	98
			FJ976552 <i>Bacillus megaterium</i> strain LCR43	98
02	BTT24P	1184	JQ039972 <i>Bacillus subtilis</i> strain YNA61	98
			HQ143570 <i>Bacillus pumilus</i> strain YT1	98
03	CTT04P	1145	JN086146 <i>Bacillus amyloliquefaciens</i> strain Rx-34	98
			JF496465 <i>Bacillus vallismortis</i> strain WA1-1	98
04	DTT07P	1174	JN084155 <i>Bacillus aryabhattai</i> strain Y8	98
			FJ620896 <i>Bacillus megaterium</i> strain XTBG34	98
05	HGT06P	957	HQ231223 <i>Bacillus</i> sp. NyZ44	98
			HQ242772 <i>Bacillus aryabhattai</i> isolate PSB59	98
06	KGT15P	931	AF270793 <i>Bacillus subtilis</i> N5	99
			JF738142 <i>Bacillus</i> sp. PSM2	99
07	STT05P	1117	FJ210844 <i>Staphylococcus saprophyticus</i> strain OTUC3	99
			HM854231 <i>Staphylococcus xylosus</i> strain KTH6-1	99
08	TVT05P	967	GQ480491 <i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i> train xf1-4	98
			HM 854231 <i>Staphylococcus xylosus</i> strain KTH6-1	97
09	VLT02P	720	JF496324 <i>Bacillus vallismortis</i> strain A1-7	98
			HQ238495 <i>Bacillus methylotrophicus</i> strain S521B-53	98
10	TGT03P	1360	HM055608 <i>Bacillus amyloliquefaciens</i> strain JS	99
			JF899261 <i>Bacillus methylotrophicus</i> strain Hk9-21	99

Figure 3.3: Phylogenetic tree for partial 16S rRNA gene sequences from 10 protein FPB isolates by using primers (37F, 1492R) showing relationships between representative strains along with related sequences retrieved from GenBank. The numbers at the nodes indicate the levels of bootstrap support (%) based on a Maximum Likelihood analysis of 100 re-sampled datasets. The scale bar indicates the phylogenetic distance corresponding to 5 changes per 100 bases.

; cluster A2 had 3 strains with high similarity (100%) as *Bacillus vallismortis* VLT02P, *Bacillus aryabhattai* HGT06P and *Bacillus subtilis* KGT15P, three strains originated from far from (more than 100 km) but they related close genetic.

Cluster B had 2 small clusters: cluster B1 with 2 strains *Bacillus megaterium* AGT08P related close with *Staphylococcus saprophyticus* STT05P and cluster B2 with 2 strains as *Bacillus subtilis* BTT24P also had high relationship with *Bacillus amyloliquefaciens* CTT04P. In general, gram-positive bacteria occupied the majority among genus *Bacillus* (8/10) and genus *Staphylococcus* (2/10).

The Polysaccharide FPB Isolates:

Among the 10 strains in the phylogenetic tree (Figure 3.4), 9 were positive-gram bacteria including 8 belonged to genus *Bacillus* (majority) and 1 was genus *Arthrobacter*; the 1 isolates was negative-gram bacteria and belonged to genus *Rhizobium*. The phylogenetic tree had 2 clusters: cluster A composed of 2 small clusters, cluster A1 with 4 strains: *Bacillus* sp. HGT09PS, *Bacillus* sp. KGT50PS, *Bacillus* sp. TVT35PS and *Bacillus* sp. BTT36PS with high similarity and originating from the same as provinces at the seaside of the Mekong Delta; cluster A2 with 2 strains: *Bacillus megaterium* AGT19PS and *Arthrobacter* sp. DTT45PS. DTTPS45PS.

Figure 3.4: Phylogenetic tree for partial 16S rRNA gene sequences from 10 polysaccharide FPB isolates by using primers (37F, 1492R) showing relationships between representative strains along with related sequences retrieved from GenBank. The numbers at the nodes indicate the levels of bootstrap support (%) based on a Maximum Likelihood analysis of 100 re-sampled datasets. The scale bar indicates the phylogenetic distance corresponding to 5 changes per 100 bases.

Table 3.3: Phylogenetic affiliation of 10 polysaccharide FPB isolates on the basis of 16S rDNA gene sequences by using BLAST programme in the GenBank database based on sequence similarity

No	Strain	Number nucleotides	of	Closest species relative	Similarity (%)
01	AGT19PS	1075		JN411325 <i>Bacillus megaterium</i> strain IARI-BK-14 EU834243 <i>Bacillus subtilis</i> strain DS13	99 99
02	BTT36PS	1171		KP992157 <i>Bacillus</i> sp. MNPK-2 JN642548 <i>Bacillus megaterium</i> strain EN2	98 98
03	CTT27PS	836		EU162025 <i>Bacillus</i> sp. PGBw5	99
04	DTT45PS	1184		HQ657321 <i>Arthrobacter</i> sp. D48 JF900051 <i>Arthrobacter</i> sp. C3-2	98 98
05	HGT09PS	1179		GQ284474 <i>Bacillus megaterium</i> strain PCWCW5 JF460759 <i>Bacillus aryabhathi</i> strain Kt10-17	99 99
06	KGT50PS	988		EU661789 <i>Bacillus</i> sp. C-18 HM027880 <i>Bacillus megaterium</i> strain RKJ 600	98 98
07	TVT35PS	949		HQ231223 <i>Bacillus</i> sp. NyZ44 FJ976550 <i>Bacillus megaterium</i> strain LCR41	99 99
08	STT37PS	1211		JN048647 <i>Rhizobium</i> sp. SYF-5 GQ181060 <i>Agrobacterium tumefaciens</i> strain BLN4	98 97
09	VLT22PS	1360		EU912461 <i>Bacillus</i> sp. SuP1 JF496312 <i>Bacillus megaterium</i> strain XAS5-1	97 97
10	TGT09PS	1239		JF496312 <i>Bacillus megaterium</i> strain XAS5-1 JN700159 <i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> strain P03	98 98

In cluster B composed of 4 strains: *Bacillus megaterium* VLT22PS and *Bacillus megaterium* TGT09PS related close relationship, they had a relationship with *Bacillus* sp. CTT27PS and all of them had an relationship with *Rhizobium* sp. STT37PS.

Effects of Environmental Factors on Flocculating Activity (%)

Three good bacterial strains of polysaccharide FPB were selected to evaluate the effects of pH, cations and optimal conditions on growth of bacteria to obtain the highest flocculating activity.

+ **pH:** At pH 6, all of three bacterial strains had the highest flocculating activities, while the flocculating activities were not same at pH 5, 7, 8 and 9 (Table 3.4).

+ **Cation:** Among 5 kinds of the cations used (CaCl_2 , NaCl, KCl, MnSO_4 , MgSO_4), the cation of CaCl_2 had the highest flocculating activities (Table 3.5). In comparison with the NaCl, the CaCl_2 was high cost and the flocculating activity was no different. Therefore, the NaCl were used to replace the CaCl_2 . Moreover, the NaCl had higher flocculating activities than the CaCl_2 at low cations concentration (0.11 to 0.12%) for all the bacterial strains (Table 3.6).

Table 3.4: Effects of pH on flocculating activity (%) of the polysaccharide FPB strains

Strain	pH				
	5	6	7	8	9
AGT19PS	60.35 b	69.02 a	63.59 b	63.51 b	62.18 b
STT37PS	61.39 b	68.80 a	68.47 a	61.53 b	66.46 a
TGT09PS	67.07 a	68.84 a	65.88 a	65.65 a	66.47 a

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Table 3.5: Effects of cations on flocculating activity (%) of the polysaccharide FPB strains

Strain	Cations				
	CaCl ₂	NaCl	KCl	MnSO ₄	MgSO ₄
	Flocculating activity (%)				
AGT19PS	68.41 a	68.40 a	52.97 c	59.44 b	50.58 c
STT37PS	70.78 a	69.72 a	53.52 c	58.47 b	53.58 c
TGT09PS	68.89 a	66.05 a	55.63 b	52.69 bc	50.16 c

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Optimal Conditions for the Growth of the FPB

To determine an optimal medium for the good growth of FPB, one type of medium should be made with adequate nutritional ingredients (carbon, nitrogen, minerals,..) for the growth of bacteria. So, an experiment was carried out to look for the best formula of medium.

The results in table 3.7 showed that 1% sucrose + 5% glutamic acid was used, the highest flocculating activities obtained in all the three bacterial strains.

To evaluate the effectiveness of the three bacterial strains, flocculating activities on water infecting many suspended solid with Kaolin of the bacterial strains was performed independently or in combination with 2 or 3 the bacterial strains. The results in Table 3.8 showed that the usage of one the strain (STT37PS) demonstrated the highest flocculating activity (%) in comparison to the combinations.

AG : AGT19PS; ST : STT37PS; TG : TGT09PS and the evaluating of the effectiveness of the three bacterial strains to wastewater in tra-fish ponds (Kaolin liquid was replaced by wastewater of the tra-fish pond), the results in Table 3.9 showed that the effectiveness of the bacterial strains for the suspended solid with Kaolin, however the flocculating activities were lower than in comparison to the suspended solid with Kaolin perhaps due to the presence of other suspended solids in the wastewater from the tra-fish ponds.

Table 3.6: Effects of NaCl and CaCl₂ on the flocculating activity (%) of the polysaccharide FPB strains.

Strain	Used level (%)	Kaolin NaCl +	Kaolin CaCl ₂ +	strain	Used level (%)	Kaolin NaCl +	Kaolin CaCl ₂ +	strain	Used level (%)	Kaolin NaCl +	Kaolin CaCl ₂ +
AGT 19PS	0.08	42.27 c.	25.00 bc	STT 37PS	0.08	74.04 abc	58.75 b	TGT 09PS	0.08	40.89 c	25.31 d
	0.09	44.79 bc	25.39 bc		0.09	72.28 c	67.66 a		0.09	43.26 c	28.20 d
	0.10	39.43 c	27.66 b		0.10	75.57 ab	69.45 a		0.10	41.04 c	36.80 c
	0.11	39.28 c	23.75 c		0.11	75.80 a	70.63 a		0.11	65.39 a	38.13 c
	0.12	64.09 a	27.34 b		0.12	75.50 ab	72.81 a		0.12	50.15 b	51.88 b
	0.20	53.29 b	61.02 a		0.20	73.35 c	72.50 a		0.20	41.58 c	63.98 a

Means within a column followed by the same letter/s are not significantly different at $p < 0$.

Table 3.7: Optimal medium for the growth of the 3 FPB strains.

Carbon sources	Nitrogen sources												Minerals sources
	Glutamic acid (5%)			Yeast extract (0.05%)			Urea (0.05%)			NH ₄ SO ₄ (0.05%)			
Glucose (1%)	54.97	60.07	64.77	52.66	47.45	51.98	54.43	51.07	41.90	52.96	46.32	44.75	Mineral applied in the treatments (red numbers)
	32.72	37.44	29.67	41.72	42.02	34.75	46.04	49.83	39.40	45.19	47.68	40.72	
	64.36	58.99	59.56	58.04	47.79	48.23	58.81	52.77	38.85	54.58	44.85	43.15	
	37.80	44.17	54.90	49.19	39.59	35.86	54.58	42.76	44.20	52.96	48.76	29.88	
Sucrose (1%)	65.82	58.14	50.73	63.51	49.21	50.31	60.59	47.00	48.58	64.97	47.74	36.97	KCl (0.5%) FeCl ₃ (0.5%) CaCl ₂ (0.5%) K ₂ HPO ₄ (0.2%) + KH ₂ PO ₄ (0.5%)
	37.49	44.85	31.83	45.42	41.40	35.23	46.50	42.08	35.58	44.65	45.31	29.26	
	69.13	70.48	68.59	64.97	56.11	56.29	55.81	50.51	51.42	66.20	50.00	42.88	
	58.43	61.20	59.21	57.43	52.94	33.84	52.19	52.77	41.07	59.89	44.06	46.42	
Starch (1%)	13.47	32.18	33.77	59.58	39.93	52.88	46.19	38.52	35.65	33.72	38.24	28.35	
	38.34	36.99	30.99	47.58	45.36	40.24	40.49	42.42	33.43	46.04	48.25	46.63	
	32.95	29.69	22.03	36.80	36.65	39.61	28.95	29.71	18.42	44.34	45.14	20.22	
	37.80	28.79	35.72	37.95	33.14	41.77	34.64	30.09	48.92	31.10	38.35	55.94	
	AG	ST	TG	AG	ST	TG	AG	ST	TG	AG	ST	TG	

Table 3.8: Effects of a single strain or combination of two strains on flocculating activity of water added suspended solids.

Strain	Flocculating activity (%)	Combination	Flocculating activity (%)
STT37PS	81.20 a	AGT19PS + TGT09PS	75.92 bc
AGT19PS	70.98 b	STT37PS + AGT19PS	73.01 cd
TGT09PS	70.14 b	STT37PS + TGT09PS	60.65 e

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Table 3.9: Effects of 3 polysaccharide FPB strains on flocculating activity of wastewater from the tra-fish ponds.

Strain	Kaolin liquid	Polluted water in tra-fish pond of OMon	Polluted water in tra-fish pond of TraVinh
STT37PS	80.33 a	53.26 a	52.14 a
AGT19PS	72.92 b	40.55 b	44.50 b
TGT09PS	72.01 b	40.55 b	41.59 c

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Application of Polysaccharide FPB for Wastewater Treatment of the Tra-Fish Ponds

The polysaccharide FPB strain STT37PS was applied to treat wastewater from the tra-fish ponds (at 3 different sites in Can Tho city) with 100-L container model. The results in Table 3.10 showed that all the strains could reduce TSS concentration (from 49 to 65%) and COD values (from 40 to 67%) after 1 day. The strain STT37PS could reduce the TSS concentration and reached less than 100 mg/L. Therefore, the strain STT37PS had potential to treat wastewater.

Table 3.10: Effects of the strain STT37PS on TSS and COD concentration in water of tra-fish from 3 different sites in CanTho city.

Site		TSS mg/L	Rate of reducti on (%)	COD mg/L	Rate of reduction (%)
CanTho 1	Regulation: QCVN 08:2008/ BTNMT, standard B1	50		30	
	Control (no bacteria)	60.50		44	
	STT37PS	21.00	65.29	26	40.91
CanTho 2	Regulation: QCVN 08:2008/ BTNMT, standard B1	50		30	
	Control (no bacteria)	124.5		49	
	STT37PS	63.50	49.00	16	67.35
CanTho 3	Regulation: QCVN 08:2008/ BTNMT, standard B1	50		30	
	Control (no bacteria)	152.50		52	
	STT37PS	64.50	57.70	29	44.23

Application of FPB for Wastewater Treatment of Fish Ponds

ThacLac fish (*Notopterus notopterus*) [Pallas, 1969] and RoDong fish (*Anabas testudineus*) (Bloch, 1792) have been feed in the Mekong popularly, the areas of fish ponds increased. Initially the area of the fish ponds of 2 districts (LongMy and ViThuy) in HauGiang province was 80 ha (2011). Until now, the area reached nearly 200 ha (2020). Besides increasing of area of ponds and fish/ponds, then erosion of soil, residues of feed, etc. had been caused an increase in the amount of waste in ponds (wastewater + pond bottom mud), polluting the environmental water and fish were attacked by pathogens (as anaerobic bacteria) easily.

Table 3.11: Physical and Chemical Characteristics of water in ThacLac pond and RoDong ponds.

Target	pH	Amoni (mg/L)	PO ₄ ³⁻ (mg/L)	TN (mg/L)	NO ₂ (mg/L)	NO ₃ (mg/L)	TP (mg/L)	TSS (mg/L)	COD (mg O ₂ /L)	BOD ₅ (mg O ₂ /L)
ThacLac	6.99	3.39	0.01	6.72	0.43	0.04	5.62	127.5	64.5	27.5
RoDong	7.45	42.5	3.90	8.40	0.07	0.03	1.92	251.5	195.5	79.5

Table 3.12: Effects of bio-floc on the physical, chemical characteristics of water in the ThacLac fish pond after 1 hour, 1 day, 2 days and 3 days.

Target	Application of bio-floc into water					Water transferred into <i>Lemna</i> pond	CV (%)	Regulation QCVN40: 2011/BTNMT
	Initiate	after 1 h	after 1 day	after 2 days	after 3 days			
pH	6.99 a	6.77 b	6.78 b	6.70 b	6.80 b	7.33 c	0.18	6-9
PO ₄ ³⁻ (mg/L)	0.01 f	0.41 e	1.13 c	1.83 b	2.77 a	0.73 d	1.73	6*
NO ₂ ⁻ (mg/L)	0.43	0.02	0.02	0.02	0.02	0.02		0.01*
NO ₃ ⁻ (mg/L)	0.04	ND	ND	ND	0.03	0.06		50*
TP (mg/L)	5.62 a	2.83 d	3.08 c	2.65 e	3.80 b	0.62 f	0.28	4

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Table 3.13: Effects of bio-floc on the physical, chemical characteristics of water in the RoDong fish pond after 1 hour, 1 day, 2 days and 3 days.

Target	Application of bio-floc into water					Water transferred into <i>Lemna</i> pond	CV (%)	Regulation QCVN 40:2011 /BTNMT
	Initiate	after 1 h	after 1 day	after 2 days	after 3 days			
pH	7.45 a	6.96 d	7.18 c	7.14 c	7.13 c	7.32 b	0.18	6-9
PO ₄ ³⁻ (mg/L)	3.90 e	68.1 b	77.1 a	38.9 d	40.1 c	0.23 f	0.26	6*
NO ₂ ⁻ (mg/L)	0.43	0.02	0.02	0.02	0.02	0.02		0.01*
NO ₃ ⁻ (mg/L)	0.04	ND	ND	ND	0.03	0.06		50*
TP (mg/L)	1.92 b	2.68 a	0.65 d	0.85 c	0.53 e	2.64 a	0.06	4

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

The results in table 3.11 showed that the quality of water in the RoDong pond achieved not in comparison to the quality of water in the ThacLac pond.

In order to remove toxic materials out of the ponds, water in ponds in farms were only replace twice one month. The water in the ponds irrigated directly to rivers and canals. Moreover, chemicals and oxygen-generating reel were applied by a few of farms but cost is high for small farm-family and economic efficiency for big farm-family. Therefore, using FPB products (Bio-floc) in the clear water environment is applied.

Application of FPB products in water treatment of the ThacLac pond were performed in after 1 hour, 1 day, 2 days and 3 days. The samples of the water in the ThacLac pond was collected to measure pH, PO_4^{3-} (mg/L), NO_2^- (mg/L), NO_3^- (mg/L), TP (mg/L) and TN, TSS, COD, BOD,... The results were presented in table 3.12.

Figure 3.5: Effects of Bio-Floc and *Lemna* sp. on TN and Amoni concentrations in water of the ThacLac fish pond.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.6: Effects of Bio-floc on TSS, COD and BOD concentrations in water of the ThacLac fish pond.

The results (Table 3.12., Figure 3.5. and Figure 3.6.) showed that concentrations of TSS, COD, BOD₅, TN, Nitrite, Nitrate, TP and PO₄³⁻ in water of bronze featherback fish pond decreased strongly after 1 hour applying biofloc and after 72 hours, all of data were lower than A standard of Regulation QCVN40:2011/BTNMT; Ammonia concentration increased from 1 to 3 days but it reduced strongly when water was moved to *Lemna* pond.

Application of FPB products in water of the RoDong fish pond in after 1 hour, 1 day, 2 days and 3 days, water in the RoDong fish pond was collected to measure pH, PO₄³⁻ (mg/l), NO₂⁻ (mg/l), NO₃⁻ (mg/l), TP (mg/l) and TN, TSS, COD, BOD... The results presented in table 3.13.

Due to water in the RoDong fish pond contained many toxic materials; concentrations of COD, BOD₅, TP, Nitrite, Nitrate in water of the RoDong fish pond decreased under A standard of Regulation QCVN40 after 3 days applying bio-floc however concentrations of Ammonia, PO₄³⁻, TN increased during 3 days after applying bio-floc and they only reduced strongly under A standard of the Regulation (QCVN40) when water was transferred to *Lemna* pond; neutral pH of water (two ponds) changed slowly.

This process was applied for water treatment of three-month old bronze featherback (ThacLac) fish and ababas (RoDong) fish ponds successfully with data under A standard of Regulation QCVN40 in a short time (3 days for bio-floc stage, 2 days for *Lemna* stage) with low cost.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.7: Effect of Bio-Floc and *Lemna* sp. on TN and Amoni concentrations in water of the RoDong fish pond.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.8: Effects of Bio-floc on TSS, COD and BOD concentrations in water of the RoDong fish pond.

Applications of FPB in Treatment of Piggery Wastewater

Raising of pigs in the Mekong Delta has been developed in family scales and farms scales strongly but the wastewater generated from the pig farms has been not treated yet and drained to rivers and canals. This is one of the causes of water source pollution in the rivers and the canals which people often use the water from these sources for cooking and other living activities daily. Therefore, the wastewater of pig farms need to be cleaned before drain to rivers and canals.

One hundred-nineteen (119) Protein FPB isolates (53.85 %) and 102 Polysaccharide FPB (46.15 %) isolates were isolated from 147 samples which collected from 13 cities and provinces in the Mekong Delta (Table 3.14).

Table 3.14: FPB isolates isolated from 13 sites in the Mekong Delta.

No	Site	Number of samples	Number of isolated isolates	
			Protein	Polysaccharide
01	KienGiang	17	12	9
02	CanTho	22	6	7
03	BenTre	21	6	12
04	TienGiang	20	15	20
05	VinhLong	12	2	6
06	TraVinh	22	7	7
07	SocTrang	22	18	4
08	DongThap	21	9	3
09	CaMau	17	12	7
10	AnGiang	16	15	13
11	HauGiang	15	6	7
12	BacLieu	11	5	4
13	LongAn	26	6	3
	Total	242	119	102

All of the FPB isolates were identified by PCR technique with primers 37F and 1479R. The PCR products were identified at 1500 bps position. Therefore they were the FPB isolates.

The FPB strains developed very well on the media from 48 - 72 h at 30°C. The colonies had round-shape, spreading, slimy, smooth, transparency, colorless or milk-color, white or yellow. Most of the colony size of the strains were small (1 – 1.5 mm in diameter), while a few strains were large (2.5 – 3 mm in diameter) (Figure 3.9).

Morphological characteristics of the FPB cells were short rod-shaped and circular. The cell had an outer mucus and viscous layer that helped to flocculate the organic and inorganic suspended particles and settle at the bottom of containers. Selection of 10 isolates had the highest flocculating activities (FA) to identify them by 16S rRNA genes sequencing.

Figure 3.9: Colonies and cells of the protein FPB isolates (above) and the polysaccharide FPB isolates (below) under SEM.

Identification of the FPB Isolates

The Protein FPB Isolates:

Eighteen isolates had the high flocculating activities were chosen to identify by PCR technique and sequencing.

The results in Table 3.15 showed that all of the 18 isolates belonged to genus *Bacillus*. The phylogenetic tree showed that the 18 protein FPB strains had 2 big clusters: Cluster A with 2 small clusters: cluster A1 had 7 strains as TIENGIANG.13, TRAVINH.1, BENTRE.31, ANGIANG.49, SOCTRANG.82, CAMAU.64 and CANTHO.5 from 7 different provinces. Among 3 strains TIENGIANG.13, TRAVINH.1, BENTRE.31 had close relationship perhaps they originated from 3 provinces nearly; 2 strains: SOCTRANG.82 and CAMAU.64 also had relationship closely and 5 sites were 5 provinces located at the seaside (Eastern Sea). Two strains ANGIANG.49 and CANTHO.5 distributed in 2 separate clades but all of them (7 strains) were identified *Bacillus megaterium*. Cluster A2 with 2 small clusters as cluster A21 with *Bacillus megaterium* KIENGIANG.32 and *Bacillus megaterium* KIENGIANG.61 related very closely, Three strains *Bacillus aryabhatai* HAUGIANG.3, BACLIEU.93 and LONGAN.21 had high relationship.

Cluster B had 2 clusters and the 6 strains were distributed belong to local site as cluster B1 with 3 strains *Bacillus megaterium* CANTHO.3, and CAMAU.64 together with *Bacillus* sp. VINHLONG.1 and cluster B2 with 3 strains *Bacillus megaterium* LONGAN.51, DONGTHAP.42 and ANGIANG.84.

Phylogenetic tree of the 18 protein FPB strains belonged to genus *Bacillus* that the species of Bacilli occupied the dominance in flocculants producing bacteria and they survived very well in wastewater piggery. Even though they distributed to local sites but they had the relationships of genome.

Table 3.15: Phylogenetic affiliation of 18 protein FPB isolates on the basis of 16S rDNA gene sequences by using BLAST programme in the GenBank database based on sequence similarity

No	Strain	Closest species relative	Similarity (%)
01	KG32P	JF496309 <i>Bacillus megaterium</i> strain XAS3-2	98
		EU834243 <i>Bacillus subtilis</i> strain DS13	98
02	KG61P	HM027880 <i>Bacillus megaterium</i> strain RKJ 60	98
		JF496300 <i>Bacillus megaterium</i> strain XA7-7-1	98
03	CT3P	JQ407796 <i>Bacillus aryabhattai</i> strain BVC13	98
		JF496312 <i>Bacillus megaterium</i> strain XAS5-1	98
04	CT5P	HQ647284 <i>Bacillus megaterium</i> strain TS58	98
		FJ823003 <i>Bacillus megaterium</i> strain gx-98	98
05	BT31P	JF820121 <i>Bacillus megaterium</i> strain PG-5-8	98
		JF496506 <i>Bacillus megaterium</i> strain WAS1-2	98
06	TG13P	JF496309 <i>Bacillus megaterium</i> strain XAS3-2	98
		JF496454 <i>Bacillus aryabhattai</i> strain EAS5-1	98
07	VL1P	FJ976552 <i>Bacillus megaterium</i> strain LCR43	98
		EU931553 <i>Bacillus megaterium</i> strain ZFJ-14	98
No	Strain	Closest species relative	Similarity (%)
08	TV1P	JN642548 <i>Bacillus megaterium</i> strain EN2	98
		HQ242772 <i>Bacillus aryabhattai</i> isolate PSB59	98
09	ST82P	HQ143579 <i>Bacillus megaterium</i> strain DZQ3-1	99
		JN411298 <i>Bacillus megaterium</i> strain IARI-AN-28	98
10	DT42P	FJ976616 <i>Bacillus megaterium</i> strain LCR107	98
		GU563347 <i>Bacillus aryabhattai</i> strain LS11	98
11	CM641P	JN903382 <i>Bacillus megaterium</i> strain W33	97
		JF899293 <i>Bacillus aryabhattai</i> strain Hc15	97
12	CM64P	FJ976544 <i>Bacillus megaterium</i> strain LCR35	98
		EU979528 <i>Bacillus megaterium</i> strain TS-1	98

13	AG49P	GU048867 <i>Bacillus megaterium</i> strain TOBCMDU-1	99
		FJ174642 <i>Bacillus megaterium</i> strain 129YG13	97
14	AG84P	FN435884 <i>Bacillus megaterium</i> isolate 13	99
15	HG3P	HM357355 <i>Bacillus megaterium</i> strain 3-1-2	99
		HM027880 <i>Bacillus megaterium</i> strain RKJ 600	98
		JF460759 <i>Bacillus aryabhattai</i> strain Kt10	98
16	BL93P	JF496310 <i>Bacillus megaterium</i> strain XAS4-7	98
17	LA21P	JF895489 <i>Bacillus megaterium</i> strain As-30	99
		FJ605385 <i>Bacillus megaterium</i> strain AceR-2	99
		JF683607 <i>Bacillus megaterium</i> strain KU1	99
18	LA51P	HQ242768 <i>Bacillus megaterium</i> isolate PSB55	99
		HQ840732 <i>Bacillus megaterium</i> strain MBFF6	99

Figure 3.10: Phylogenetic tree for partial 16S rRNA gene sequences from the 18 protein FPB isolates by using primers (37F, 1492R) showing relationships between representative strains along with related sequences retrieved from GenBank. The numbers at the nodes indicate the levels of bootstrap support (%) based on a Maximum Likelihood analysis of 100 re-sampled datasets. The scale bar indicates the phylogenetic distance corresponding to 5 changes per 100 bases.

The Polysaccharide FPB Isolates:

Among the 16 strains in phylogenetic tree (Figure 3.11) belonged to genus *Bacillus* (majority) (10/16), genus *Klebsiella* (3/16), *Ochrobactrum anthropi* (2/16) and genus *Sphingobacterium* (1/16). They included 62.5 % positive-gram bacteria [10/16] (majority genus *Bacillus*) and 37.5 % negative-gram bacteria were [6/16].

Table 3.16: Phylogenetic affiliation of 16 polysaccharide FPB isolates on the basis of 16S rDNA gene sequences by using BLAST programme in the GenBank database based on sequence similarity.

No	Strain	Closest species relative	Similarity (%)
01	KG12S	JQ407796 <i>Bacillus aryabhattai</i> strain BVC13	98
		JF496312 <i>Bacillus megaterium</i> strain XAS5-1	98
02	CT63S	HQ908708 <i>Bacillus aryabhattai</i> strain F77063	99
		HQ242772 <i>Bacillus aryabhattai</i> isolate PSB59	99
03	BT1S	JF496290 <i>Bacillus aryabhattai</i> strain XA6-1	97
		AY167862 <i>Bacillus megaterium</i> strain SAFR-038	97
04	BT2S	JN411575 <i>Bacillus megaterium</i> strain S4	98
		FR821658 <i>Bacillus megaterium</i> partial strain 108X	98
05	TG21S	FJ3741261 <i>Ochrobactrum anthropi</i> strain P23	97
		DQ417342 <i>Ochrobactrum anthropi</i> strain WZR	97
06	TG32S	AY917134 <i>Ochrobactrum anthropi</i> isolate CYP2004	98
		KC252888 <i>Ochrobactrum anthropi</i> strain R058	97
07	VL4S	EF426437 <i>Sphingobacterium</i> sp. 1.3	98
		JX035964 <i>Sphingobacterium multivorum</i> strain GZT-2	97
08	TV43S	JQ308544 <i>Bacillus amyloliquefaciens</i> strain APS3	98
		JQ308585 <i>Bacillus amyloliquefaciens</i> strain YPA6	98
09	ST71S	HM753596 <i>Bacillus subtilis</i> strain AVT-KSU309	99
		JQ308552 <i>Bacillus subtilis</i> strain FPA3	98
10	DT16S	GU272350 <i>Klebsiella</i> sp. LP1MK	98
		FJ490056 <i>Klebsiella pneumoniae</i> strain F2	98
11	DT42S	JQ698335 <i>Bacillus amyloliquefaciens</i> strain N-6	98
		JF935098 <i>Bacillus subtilis</i> strain ChST3.4	98
12	CM31S	FJ174605 <i>Bacillus megaterium</i> strain 157XG76	97
		GU048868 <i>Bacillus megaterium</i> strain TOBCMDU-2	97
13	AG75S	GU272365 <i>Klebsiella</i> sp. TP1MC (KOREA)	99
		FJ490057 <i>Klebsiella pneumoniae</i> strain F3	99
14	HG1S	GU272365 <i>Klebsiella</i> sp. TP1MC	97
		JX435602 <i>Klebsiella pneumoniae</i> strain zg2010	97
15	BL5S	KC191571 <i>Bacillus methylotrophicus</i> strain CC	97
		GU122948 <i>Bacillus amyloliquefaciens</i> strain DBT3SC3	97
16	LA1S	HQ670558 <i>Bacillus megaterium</i> strain AIMST	97
		JQ312015 <i>Bacillus aryabhattai</i> strain AIMST	97

Figure 3.11: Phylogenetic tree for partial 16S rRNA gene sequences from the 16 polysaccharide FPB isolates by using primers (37F, 1492R) showing relationships between representative strains along with related sequences retrieved from GenBank. The numbers at the nodes indicate the levels of bootstrap support (%) based on a Maximum Likelihood analysis of 100 re-sampled datasets. The scale bar indicates the phylogenetic distance corresponding to 5 changes per 100 bases.

The phylogenetic tree with the 16 polysaccharide FPB strains (Figure 3.11) divided to 2 big clusters. Cluster C composed 11 strains with 2 small clusters; cluster C1 had 7 strains: *Ochrobactrum anthropi* TIENGIANG 32S, *Bacillus megaterium* CAMAU 31S, *Klebsiella* sp., HAUGIANG 1S, *Sphingobacterium* sp. VL4S and *Bacillus methylotrophicus* 6S, had related relationship close and they located cluster C11; and cluster C12 with 2 strains as *Bacillus amyloquelaciens* TRAVINH 43S and *Bacillus aryabhattai* BENTRE 1S had high relationship. While cluster C2 with 4 strains *Klebsiella* sp.

DONGTHAP 1S, *Bacillus megaterium* LONGAN 1S, *Bacillus megaterium* BENTRE 2S, and *Bacillus subtilis* SOCTRANG 71S, they had the high similarity because 4 provinces located close together. The strains in cluster D1 were *Klebsiella* sp. ANGIANG 7S, and *Ochrobactrum anthropi* TIENGIANG 21S. Cluster D2 composed of 3 strains: *Bacillus aryabhatai* KIENGIANG 12S and *Bacillus amyloliquefaciens* DONGTHAP 42S had relationship very closely and both strains related with *Bacillus aryabhatai* CANTHO 63S.

Effects of Environmental Factors on Flocculating Activity (%)

+ pH

Using the Kaolin suspension for determination the flocculating activities of the bacterial strains showed that (Figure 3.12): At different pH values, the flocculating activities were different. The flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryabhatai* KG12S were the highest (83.1 %, 83.2 %, respectively) at pH 6 and significant different to the other pH values. So, the pH 6 was used for the next experiments.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.12: Effects of pH on flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryabhatai* KG12S.

+ Temperature

The flocculating activities of the *Bacillus megaterium* LA51P and *Bacillus aryabhatai* KG12S were measured at different incubation temperatures from 20°C to 50°C. The results in Figure 3.13 showed that both the strains had the highest flocculating activities at 32°C. At 50°C, the flocculating activities were very low.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.12: Effects of pH on flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S.

Figure 3.13: Effects of temperatures on the flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S.

+ The growth of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S

The bacterial growth curves of both the strains in medium were the same (Figure 3.14). The growth rates were increased from 24 h to 48 h and reached the highest at 96 h (the log phase). From 96 h to 168 h, the growth did not change (the stationary phase).

+ Interactions between time, pH and temperature for the 2 strains

Analysis and construction of multi-variance regression equation about the interactions of 3 factors including time, pH and temperature by Statgraphics centurion XVI software. Based on the equation identified the optimal treatments for environmental factors to multiply the biomass of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S strains reached to the highest flocculating activities.

Together with pH, two factors as time and temperature affected to the flocculating activities clearly. The results in Table 3.17 showed that the interactions between pH, time and temperature affected on the flocculating activities of the 2 strains.

Figure 3.14: The growth curves of the 2 strains overtime (24 to 168 h).

For *Bacillus megaterium* LA51P, at the levels of time (120 h) and pH (6), the flocculating activities was the highest and did not differ significantly at 3 levels of temperatures (30, 32 and 34°C). Therefore, this train was multiplied at the 3 levels of temperatures with fixing pH 6 and 120 h.

In the contrary, *Bacillus aryhadtai* KG12S had the highest flocculating activity at the conditions of pH 6, 120 h and 34 °C. Therefore, the medium, time (120 h) and temperature (34°C) of culturing was the same for both the strains.

To reach the highest flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S, the interactions of three factors as time, pH and temperature were performed by the Statgraphics centurion XVI software to determine the optimal environmental factors for multiply of them.

Multi-variance regression equation about interactions of the 3 factors as time, temperature and pH to the flocculating activity of *Bacillus megaterium* LA51P.

Flocculating activity (%) = - 371.622 + 10.0885 * Temperature + 5.85269 * pH + 4.22472 * Time – 0.146296 * Temperature * Temperature – 1.23407 * pH * pH – 0.0186519 * Time * Time + 0.0163194 * Temperature * pH + 0.00144097 * Temperature * Time + 0.0978819 * pH * Time – 0.00106771 * Temperature * pH * Time

With the multi-variance regression equation was presented above, replacing of Time = 120 h, pH = X = (5 – 7) and temperature = Y = (30 - 34) and the Statgraphics centurion XVI software was used to made a graph of surface plotting and graph of contour, with the equation as following:

Table 3.17: Effects of time, pH and temperature on the flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S.

Strain	Time (h)	pH			Temper ature (°C)
		5	6	7	
<i>Bacillus megaterium</i> LA51P [LSD.01= 6.61]	96	69.78	68.18	66.56	30
		70.63	69.02	67.17	32
		70.38	70.72	66.89	34
		83.19	85.18	80.34	30
	120	85.87	85.65	81.12	32
		85.67	86.09	81.08	34
	144	75.43	76.32	78.89	30
		76.98	77.89	78.91	32
		75.18	78.09	77.97	34
	96	73.12	76.18	72.19	30
		71.18	77.42	72.31	32
		72.34	75.19	71.23	34
		82.34	84.56	82.98	30

KG12S [LSD.01= 2.78]	120	83.19	83.98	80.13	32
		81.18	85.13	79.17	34
		76.18	78.93	73.13	30
	144	75.68	79.14	73.09	32
		74.18	78.69	72.17	34

Flocculating activity (%) = - 371,622 + 10.0885 * Y + 5.85269 * X + 4.22472 * 120 – 0.146296 * Y * Y – 1.23407 * X * X – 0.0186519 * 120 * 120 + 0.0163194 * Y * X + 0.00144097 * Y * 120 + 0.0978819 * X *120 – 0.00106771 * Y * X *120

From the Graph of surface plotting and graph of contour in Figure 3.15 was used to made when replacing of TIME = 120 hour, pH = X (5 - 7) and TEMPERATURE = Y (30 - 34) had the highest flocculating activity at 33°C and pH (5.7). Replacing of TEMPERATURE = 33°C, TIME = X (96 - 144) and pH = Y (5 – 7) into the regression equation, the Statgraphics centurion XVI software made a graph of surface plotting and graph of contour with the equation as following:

Figure 3.15: Graph of surface plotting and graph of contour of the flocculating activity to TIME = 120 hour, pH = X (5 - 7) and TEMPARATURE = Y (30 - 34).

Flocculant-activity (%) = - 371.622 + 10.0885 * 33 + 5.85269 * Y + 4.22472 * X – 0.146296 * 33 * 33 – 1.23407 * Y * Y – 0.0186519 * X * X + 0.0163194 * 33 * Y + 0.00144097 * 33 * X + 0.0978819 * Y *X – 0.00106771 * 33 * Y * X

Figure 3.16: Graph of surface plotting and graph of contour of flocculating activity of temperature = 33 °C, Time = X (96 - 144) and pH = Y (5 - 7)

From the Graph of surface plotting and graph of contour of the flocculating activity in Figure 3.19 was made from the regression equation when replacing of TEMPERARE = 29°C, TIME = X (96 - 144) and pH = Y (5 - 7), showed that 2 factors: starch = X = 124 hour and pH = Y = 5.7 had the best effective flocculating activity.

Optimal conditions for the Growth of Flocculants Producing Bacteria

To determine an optimal medium for the good growth of FPB, one type of medium should be made with adequate nutritional ingredients (carbon, nitrogen, minerals,...) for the growth of bacteria. So, an experiment was carried out to look for the best formula of medium. The result in Table 3.18 showed that the medium containing 1 % sucrose and 5 % glutamic acid was an optimal medium and gave the highest flocculating activities for all the three strains.

Two treatments such as the first one included Starch (1 %), glutamate (5 %), CaCl_2 (0.5 %) and the second one included Starch (1%), glutamate (5%), CaCl_2 (0.75%) had the highest flocculating activities (93.68%, 95.46%, respectively) and differed from the others significantly (Table 3.19). While other treatments had the lowest flocculating activities (43.56 % and 44.19%) including the third one included Starch (1.5%), glutamate (2.5%), CaCl_2 (0.5%) and the four one included starch (1.5%), glutamate (7.5%), CaCl_2 (0.25%).

Table 3.18: The optimal medium for the growth of *Bacillus megaterium* LA.51P and *Bacillus aryhadtai* KG12S.

Strain	Carbon sources	Nitrogen sources				Mineral sources	
		Glutamic acid (5%)	Yeast extract (0.05%)	Urea (0.05%)	NH ₄ SO ₄ (0.05%)		
<i>Bacillus megaterium</i> LA.51 LSD .01 = 2.72	Glucose (1%)	79.35	57.89	11.40	50.63	MgSO ₄ (0.5 %)	
		65.33	49.96	9.33	51.23	CaCl ₂ (0.5 %)	
		60.16	55.17	10.98	49.06	FeCl ₃ (0.5 %)	
		63.86	59.44	13.77	52.38	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)	
	Sucrose (1%)	60.84	53.05	40.80	55.48	MgSO ₄ (0.5 %)	
		58.13	46.08	33.88	53.89	CaCl ₂ (0.5 %)	
		54.67	44.42	32.13	56.81	FeCl ₃ (0.5 %)	
		50.27	45.38	33.84	57.87	K ₂ HPO ₄ (0.2%) + KH ₂ PO ₄ (0.5%)	
	Starch (1%)	65.62	73.59	77.71	60.34	MgSO ₄ (0.5 %)	
		86.12	71.33	77.86	59.67	CaCl ₂ (0.5 %)	
		65.09	74.18	75.67	60.23	FeCl ₃ (0.5 %)	
		63.69	76.31	77.64	57.47	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)	
	<i>Bacillus aryhadtai</i> KG12S LSD .01 = 3.84	Glucose (1%)	68.17	44.16	46.18	75.17	MgSO ₄ (0.5 %)
			71.34	46.18	43.18	46.18	CaCl ₂ (0.5 %)
			73.41	75.24	75.12	69.08	FeCl ₃ (0.5 %)
			88.36	68.31	58.38	79.15	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)
Sucrose (1%)		62.34	66.19	51.72	70.02	MgSO ₄ (0.5 %)	
		66.18	68.16	49.17	69.53	CaCl ₂ (0.5 %)	
		69.98	69.09	52.18	66.17	FeCl ₃ (0.5 %)	
		79.95	55.18	53.85	69.09	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)	
Starch (1 %)		68.17	42.39	42.14	71.23	MgSO ₄ (0.5 %)	
		65.87	50.18	68.13	70.21	CaCl ₂ (0.5 %)	
		71.24	54.14	71.28	72.18	FeCl ₃ (0.5 %)	
		81.42	76.16	79.14	78.19	K ₂ HPO ₄ (0.2%) + KH ₂ PO ₄ (0.5 %)	
CV % = 2.76							

Table 3.19: Effects of glutamate, glucose and minerals on flocculating activity of *Bacillus megaterium* LA51P.

	Carbon sources	Nitrogen sources			Inorganic mineral sources (%)
		Glutamate (2.5 %)	Glutamate (5.0 %)	Glutamate (7.5 %)	
LA51P LSD .01 = 13.05 CV % = 8.06	Starch (0.5 %)	59.44	57.89	80.40	CaCl ₂ (0.25 %)
		59.12	71.95	86.28	CaCl ₂ (0.50 %)
		69.75	70.70	87.62	CaCl ₂ (0.75 %)
	Starch (1.0 %)	86.43	90.19	72.86	CaCl ₂ (0.25 %)
		86.85	93.68	54.43	CaCl ₂ (0.50 %)
		56.57	95.46	71.04	CaCl ₂ (0.75 %)
	Starch (1.5 %)	59.14	56.87	44.19	CaCl ₂ (0.25 %)
		43.56	60.51	75.77	CaCl ₂ (0.50 %)
		52.61	61.33	59.65	CaCl ₂ (0.75 %)

Table 3.20: Effects of glutamate, glucose and minerals on flocculating activity of *Bacillus aryhadtai* KG12S.

	Carbon source	Nitrogen sources			Inorganic minerals sources (%)
		Glutamate (2.5 %)	Glutamate (5.0 %)	Glutamate (7.5 %)	
KG12S LSD .01 = 13.05 CV % = 8.06	Glucose (0.5 %)	60.18	66.72	63.18	K ₂ HPO ₄ (0.1 %) + KH ₂ PO ₄ (0.25 %)
		62.31	67.18	64.58	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)
		65.98	68.34	62.19	K ₂ HPO ₄ (0.4 %) + KH ₂ PO ₄ (1 %)
	Glucose (1.0 %)	76.24	87.88	81.19	K ₂ HPO ₄ (0.1 %) + KH ₂ PO ₄ (0.25 %)
		77.11	88.36	82.18	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)
		75.98	91.35	85.47	K ₂ HPO ₄ (0.4 %) + KH ₂ PO ₄ (1 %)
	Glucose (1.5 %)	70.13	75.18	78.19	K ₂ HPO ₄ (0.1 %) + KH ₂ PO ₄ (0.25 %)
		71.26	77.98	79.08	K ₂ HPO ₄ (0.2 %) + KH ₂ PO ₄ (0.5 %)
		69.98	76.28	80.01	K ₂ HPO ₄ (0.4 %) + KH ₂ PO ₄ (1 %)

Table 3.21: Multi-variance regressive equation of interactions of 3 nutrient factors of *Bacillus megaterium* LA51P.

Equations	Starch (%)	Glutamate (%)	CaCl ₂ (%)
Flocculating activity (%) = - 24.6473 + 141.702 * starch + 15.225 * Glutamate + 56.9652 * CaCl₂ - 55.7148 * starch * starch - 0.924148 * Glutamate * Glutamate - 22.8237 * CaCl₂ * CaCl₂ - 7.80511 * starch * Glutamate - 61.5133 * starch * CaCl₂ - 3.79022 * Glutamate * CaCl₂ + 10.0467 * starch * Glutamate * CaCl₂			
Flocculating activity (%) = - 23.7208 + 33.6744 * Y + 15.0676 * X + 134.774 * 1 - 57.9985 * 1 * 1 - 0.992919 * X * X - 15.358 * Y * Y - 5.94967 * 1 * X - 32.193 * 1 * Y - 1.23137 * X * Y + 5.27257 * 1 * X * Y	1	X (2.5 -7.5 %)	Y (0.25 -0.75 %)
Flocculating activity (%) = - 23.7208 + 33.6744 * 0.9 + 15.0676 * Y + 134.774 * X - 57.9985 * X * X - 0.992919 * Y * Y - 15.358 * 0.9 * 0.9 - 5.94967 * X * Y - 32.193 * X * 0.9 - 1.23137 * Y * 0.9 + 5.27257 * X * Y * 0.9	X (0.5 -1.5 %)	Y (2.5 - 7.5 %)	0.9
Note: X, Y, Z were the changed variances of the factors (%) including starch, glutamate and CaCl ₂			

***Bacillus megaterium* LA51P:**

The multi-variance regression equation of interactions of 3 factors as starch, glutamate and minerals to the flocculating activity of *Bacillus megaterium* LA51P (in Table 3.21).

Replacing of starch $t = 1\%$, glutamate = X (2.5 - 7.5 %) and $\text{CaCl}_2 = Y$ (0.25 - 0.75 %) showed that 2 factors glutamate = X = 6.5 % and $\text{CaCl}_2 = Y = 0.9\%$ had the highest flocculating activities. From the above results, replacing of $\text{CaCl}_2 = 0.9\%$, starch = X (0.5 - 1.5%) and glutamate = Y (2.5 - 7.5%) into regressive equation showed that 2 factors: starch = X = 0.85 % and glutamate = Y = 6.6 % had the highest biomass of bacteria.

Therefore, the medium contained 0.85% starch, 6.6 % glutamate and 0.9 % CaCl_2 were used to multiply for biomass of *Bacillus megaterium* LA51P and reached the highest flocculating activities. The formula of the medium was used for the further experiments for flocculating activity of *Bacillus megaterium* LA51P and the evaluation efficiency of piggery wastewater treatment after biogas system.

***Bacillus aryhadtai* KG12S:**

In Table 3.20, combinations of 1% glucose, 5% glutamic acid and K_2HPO_4 (0.2%) + KH_2PO_4 (0.5%) in the medium had the highest flocculating activities. However, finding of the optimal conditions for the growth of *Bacillus aryhadtai* KG12S, an experiment was carried out to select the best nutrient sources (nitrogen, carbon, and minerals,...).

The results in Table 3.20 showed that the medium contained 1% glucose + 5% glutamate + K_2HPO_4 (0.4 %) + KH_2PO_4 (1%) had the highest flocculating activities.

The above results were used to determine the optimal medium for *Bacillus aryhadtai* KG12S the multi-variance regressive equation by the Statgraphics centurion XVI software. The results of the analysis showed that the flocculating activity (%) was correlated with 10 variances, regression equation:

$$\text{Flocculating activity (\%)} = -5.225 + 114.399 * \text{Glucose} + 9.156 * \text{Glutamate} + 16.367 * \text{minerals} - 51.723 * \text{Glucose} * \text{Glucose} - 0.829 * \text{Glutamate} * \text{Glutamate} - 2.531 * \text{minerals} * \text{minerals} + 0.186 * \text{Glucose} * \text{Glutamate} - 10.136 * \text{Glucose} * \text{minerals} - 1.775 * \text{Glutamate} * \text{minerals} + 1.767 * \text{Glucose} * \text{Glutamate} * \text{minerals}$$

From the multi-variance regression equation, replacing of glucose = 1 %, glutamate = X (2.5 – 7.5 %) and $\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4 = \text{Y}$ (0.35 – 1.4 %), the results from the Statgraphics centurion XVI software with graph of surface plotting (Figure 3.20) and contour equation (Figure 3.1) with the equation as following:

$$\text{Flocculating activity (\%)} = -5.22457 + 114.399*1 + 9.15619*x + 16.3672*y - 51.723*1*1 - 0.829452*x*x - 2.53061*y*y + 0.186333*1*x - 10.1361*1*y - 1.77456*x*y + 1.76748*1*x*y$$

From the Graph of surface plotting and Graph of contour equation were made from the regression equation when replacing of glucose = 1 %, glutamate = X (2.5 – 7.5 %) and minerals $\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4 = \text{Y}$ (0.35 – 1.4 %) showed that 2 factors as glutamate = X = 5.7 % and $\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4 = \text{Y} = 1.2$ % had the highest flocculating activities.

Figure 3.17: Graph of surface plotting of flocculating activity to glucose = 1%, glutamate = X (2.5 – 7.5 %) and minerals $\text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4 = \text{Y}$ (0.35 – 1.4 %)

Figure 3.18: Graph of contour equation of flocculating activity to glucose = 1 %, glutamate = X (2.5 – 7.5 %) and minerals $K_2HPO_4 + KH_2PO_4 = Y$ (0.25 – 1.4 %)

From the above results, replacing of glutamate = Y (2.5 – 7.5 %), glucose = X (1.5 – 2.5 %) and $K_2HPO_4 + KH_2PO_4 = 1.2$ into the regression equation, the Statgraphics centurion XVI software made graph of surface plotting (Figure 3.19) and graph of contour (Figure 3.20) with equation as:

Flocculating activity (%) = $- 5.22457 + 114.399 \cdot x + 9.15619 \cdot y + 16.3672 \cdot 1.2 - 51.723 \cdot x \cdot x - 0.829452 \cdot y \cdot y - 2.53061 \cdot 1.2 \cdot 1.2 + 0.186333 \cdot x \cdot y - 10.1361 \cdot x \cdot 1.2 - 1.77456 \cdot y \cdot 1.2 + 1.76748 \cdot x \cdot y \cdot 1.2$

Figure 3.19: Graph of surface plotting of flocculating activity to minerals = 1.2%, glucose = X (0.5 - 1.5%) and glutamate = Y (2.5 - 7.5%).

Figure 3.20: Graph of contour equation of flocculating activity to minerals = 1.2%, glucose = X (0.5 - 1.5%) and glutamate = Y (2.5 - 7.5%).

From the Graph of surface plotting and the Graph of contour equation (Figure 3.19 and 3.20) were made from regression equation when replacing of minerals = 1.2%, glucose = X (0.5 - 1.5%) and glutamate = Y (2.5 - 7.5%), showed that 2 factors as glucose = X = 1.12% and glutamate = Y = 5.7% had the highest biomass of *Bacillus aryhadtai* KG12S.

Therefore, the medium which was supplemented with 1.12% glucose, 5.7% glutamate and 1.2% minerals (K_2HPO_4 (0.4%) + KH_2PO_4 (0.8%)) to multiply biomass of *Bacillus aryhadtai* KG12S had the highest flocculating activity. The formula of medium was used to further experiments for optimization of bio flocculants producing by *Bacillus aryhadtai* KG12S and the evaluation of effectiveness for piggery wastewater treatment after biogas system.

Minerals Supplement:

In the previous experiment, the Kaolin suspension (control) as a kind of mineral was used in the experiment of evaluation of flocculating activity of the FPB strains. This experiment used many kinds of mineral to replace the kaolin suspension in the evaluation of flocculating activity.

The results in Table 3.22 showed that CaCl_2 was a kind of mineral which supported flocculating activity. The flocculating activity reached 91.87%.

Table 3.22: Effects of the other minerals on flocculating activity of water added suspended solids

Minerals	KCl	NaCl	CaCl ₂	MgSO ₄	MnSO ₄	FeCl ₃	Al ₂ (SO ₄) ₂	Kaolin
Flocculating activity (%)	70.18 d	66.24 e	91.87 a	86.23 b	83.12 c	88.21 b	70.18 d	21.12 f
LSD .01 = 2.18 ; CV =1.2%								

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Table 3.23: Interactions of time and bacterial population of the 2 strains after they were optimized.

Time (h)	<i>Bacillus megaterium</i> LA51P			<i>Bacillus aryhadtai</i> KG12S		
	Flocculating activity (%)	Population CFU/mL	OD 660	Flocculating activity (%)	Population (CFU/mL)	OD 660
0	0.00	6.00×10^3	0.11	0.00	6.00×10^3	0.12
24	20.96	5.00×10^7	1.42	20.96	5.30×10^7	1.42
48	80.63	3.67×10^9	1.55	80.63	4.00×10^9	1.55
72	88.89	6.67×10^9	1.66	88.89	6.67×10^9	1.65
96	94.38	7.67×10^9	1.69	96.98	6.00×10^9	1.67
120	94.18	6.00×10^9	1.61	96.18	5.33×10^9	1.60
144	94.08	4.00×10^9	1.57	94.08	4.00×10^9	1.56
168	91.01	6.67×10^8	1.48	92.01	6.67×10^8	1.47

The results in Table 3.23 showed that time (96 h) and OD660 at 1.67 – 1.69 had the highest population for both strains multiplied in the medium. These results were used for the next experiments.

Study on Bacteria Levels on Flocculating Activity:

At bacterial level of 0.2% used, the flocculating activity was the highest. (Figure 3.21)

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$.

Means within a column followed by the same letter/s are not significantly different at $p < 0.01$

Figure 3.21: Effects of bacteria levels (%) to flocculating activities of *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S

Interactions between *Bacillus megaterium* LA51P and *Bacillus aryhadtai* KG12S:

When multiplying of the 2 strains in the fermented container, the interactions between the 2 strains affected to the growth of the flocculating activities and population of FPB isolates and population.

The results in Table 3.23 showed that both the strains were not against each other or they did not compete each other and they grew parallel.

Application of FPB Strains for Wastewater Piggery after Biogas System

Effects of Ph:

Due to pH of wastewater piggery collected from other sites therefore pH varied from pH 3.52 to pH 8.13 and the FPB isolates also developed very well at the suitable pH values. Therefore, an experiment was carried out to find suitable ranges of pH for the growth of the 2 strains.

The results in Figure 3.22 showed that at pH from 5.45 to 7.35, both the strains developed very well. The pH values at near neutral (6 – 7) were suited for the growth of the FPB isolates.

Effects of Minerals Supported Flocculating:

In reality, PAC and FeCl_3 were used as minerals for supporting flocculating and replacing of Kaolin.

Figure 3.22: Interactions between pH of medium and the FPB strains.

Besides, supplementary of minerals were necessary for flocculating activity of the FPB strains in wastewater piggery treatment. In the farm-yard, farms often applied PAC and/or FeCl_3 (as support to flocculation agent) into wastewater treatment.

Figure 3.23: Effects of minerals on flocculating activity.

Figure 3.24: Effects of combination of PAC and FeCl₃ on flocculating activity

Note: P1: *Bacillus megaterium* LA51P; P2: *Bacillus megaterium* TG13P; S1: *Bacillus aryabhatai* KG12S and S2: *Klebsiella pneumoniae* ST71S

The results in Figure 3.23 and 3.24, combination of FeCl₃ (0.5 g/l) and PAC (0.5 g/L) plus bacterial level (2 ml/L) were used in the next experiments.

Application of the FPB Strains on Wastewater Piggery Treatment: 10-L Container

The four strains were applied in this experiment together with positive control (PAC 0.5 g/L; FeCl₃ 0.5 g/L), negative control (no bacteria no PAC) in wastewater piggery treatment at pH 6, OD550 = 1.261 plus 0.2% bacteria liquid, the results were presented at Figure 3.25 and Figure 3.26.

Figure 3.25: Effects of the 4 FPB strains on piggery wastewater treatment.

Figure 3.26: Effects of the 4 FPB strains on flocculating rates (%) of piggery wastewater.

Note: P1: *Bacillus megaterium* LA51P; P2: *Bacillus megaterium* TG13P; S1: *Bacillus aryabhattai* KG12S; S2: *Klebsiella pneumoniae* ST71S; P1S1; P1S2; P2S1; P2S2 were treatments of combination of the 2 strains.

The results in Figure 3.25 showed that the flocculating activities of the treatment P1 (*Bacillus megaterium* LA51P), the treatment P1S1 (*Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S) and treatment S1 (*Bacillus aryabhattai* KG12S) were 80.66, 72.36 and 70.28 %, respectively. The samples of three treatments were analysed together with positive control and negative control.

The results in Table 3.24 showed that the combination of the 2 strains could reduce TSS concentration and TP concentration strongly. However, the single strain reduced COD (>50%), TN (>40%) and TKN (>40%) concentrations but still not reached the standard of the Regulation (QCVN 40_2011/BTNMT).

Table 3.24: Effects of *Bacillus megaterium* LA51P, *Bacillus aryabhattai* KG12S) and combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S on wastewater piggery treatment after biogas system.

Target	Control Biogas*	Flocculating activity (%)			Regulation QCVN 40_2011/BTN MT	
		KG12 S**	LA51P**	LA51P_KG12S**	A	B
TSS (mg/L)	153.50	50.33	28.83	18.67	20	40
COD (mg/L)	1104.00	542.67	310.33	513.00	75	150
TKN (mg/L)	311.02	69.12	104.61	136.83	5	10
TN (mg/L)	369.86	92.47	126.09	153.18	50	100
TP (mg/L)	4.54	0.66	0.68	0.38	4	6

*one replicates, **3 replicates

Applications of the FPB Strains on Wastewater Piggery Treatment: 100-L Container

In bigger volume (100-L), two treatments as *Bacillus megaterium* LA51P and combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S were used in this study together with controls (positive and negative).

Application of *Bacillus megaterium* LA51P into wastewater treatment, the results showed that concentrations of BOD₅, COD, TN, TSS, TP and TKN reduced in comparison to negative control were 81.46 %, 87.61 %, 84.05 %, 86.11 % and 80.42 %, respectively.

Table 3.25: Effects of *Bacillus megaterium* LA51P and combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S on wastewater piggery after biogas system (100-L).

Target	Negative Control	Control PAC	LA51 P	LA51 P+ KG12 S	Regulation QCVN 40_2011/BTNMT	
					A	B
TSS (mg/L)	1975.00	1116.67	226.33	274.33	50	100
COD (mg/L)	2314.00	1540.00	913.33	286.67	75	150
BOD ₅ at 20 °C (mg/L)	1025.00	680.00	386.67	190.00	30	50
TN (mg/L)	368.93	335.31	152.24	58.84	20	40
TKN (mg/L)	198.01	196.14	81.26	63.20	5	10
TP (mg/L)	34.29	23.17	4.10	6.71	4	6

However combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S also had good efficiency for wastewater treatment (Table 3.25). Therefore, combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S was used for the study at volume of 1 m³ and 40 m³.

Application of the FPB Strains on Wastewater Piggery Treatment: 1 m³

The experiment was designed in plastic containers (>1.000 L) (Figure 3.27). Application of combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S together with 2 controls.

Before treatment with bacteria After 24 h
Figure 3.27: The experiment designed in the plastic containers (>1000 L).

Application of combination of the 2 strains reduced concentrations of BOD₅, COD and TSS in wastewater piggery in comparison to negative control were 82%, 78.83% and 66.46%, respectively.

Table 3.26: Effects of combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S on wastewater piggery treatment (after biogas system) (1000-L).

Target	Negati ve contro l*	Positive Control*	LA51P KG12S** +	Regulation QCVN 40_2011/BTNMT	
				A	B
TSS (mg/L)	81.50	125.00	27.33	50	100
COD (mg/L)	403.00	289.00	85.33	75	150
BOD ₅ at 20 °C (mg/L)	200.00	145.00	36.00	30	50
TN (mg/L)	88.73	39.23	70.61	20	40
TKN (mg/L)	72.85	31.76	40.37	5	10
TP (mg/L)	0.48	17.69	3.06	4	6

*one replicates, **3 replicates

Application of the FPB Strains on Wastewater Piggery Treatment: 50 m³

Three ponds were made to contain piggery wastewater (Figure 3.28), every pond contained over 50 m³ wastewater and bacteria liquid.

Figure 3.28: The experiment designed in the ponds contained >40 m³ piggery wastewater

After 24 h, wastewater divided into 2 parts: supernatant with height 0.8 m and bottom mud approximately 0.2 m (Figure 3.27). The supernatant was collected to analyze. The bottom mud were moved to small material-contain pond, dried slowly and then applied to Mango trees (very good). The results of analyzing of

supernatant samples were presented in Table 3.27. Concentrations of BOD₅, COD and TSS reduced in comparison to negative control were 93.67 %, 92.51 % and 82.48 %, respectively; and to positive control (PAC (1g/L) were 92.40 %, 90.79 % and 86.89 %, respectively. Concentrations of BOD₅, COD and TSS reached to A standard of the regulation (QCVN_40/2011/BTNMT).

Thus, the FPB strains (combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S strains) for wastewater piggery treatment (after biogas system) in the first stage (1 h), the toxic materials in wastewater depleted gradually. After that, the wastewater was applied with heterotrophic nitrogen removal bacteria and poly-P accumulating bacteria to treat completely and then drained to rivers/canals.

Table 3.27: Effects of combination of *Bacillus megaterium* LA51P and *Bacillus aryabhattai* KG12S strains for wastewater piggery treatment (after biogas system).

Target	Negative control*	Positive Control *	LA51P + KG12S**	Regulation QCVN 40_2011/BTNMT	
				A	B
TSS (mg/L)	137.00	183.00	24.00	50	100
COD (mg/L)	501.00	407.00	37.50	75	150
BOD ₅ at 20 °C (mg/L)	300.00	250.00	19.00	30	50
TN (mg/L)	12.59	2.42	3.09	20	40
TKN (mg/L)	31.94	161.96	18.50	5	10
TP (mg/L)	35.87	194.46	20.74	4	6

Conclusion

Isolation of 389 FPB isolates including 154 Protein FPB isolates and 235 Polysaccharide FPB isolates from polluted water in tra-fish ponds of 10 provinces and 119 FPB isolates including Protein FPB isolates and 102 Polysaccharide FPB isolates from 147 samples collected wastewater piggery from 13 cities and provinces in the Mekong Delta.

Identification of 54 isolates had the highest flocculant activity by PCR 16S rRNA showed that Bacilli occupied majority with more 80%, 20% were negative-gram bacteria,

Selected 4 strains (2 were Protein FPB and 2 were Polysaccharide FPB) for wastewater treatment, the results received that application 2 strains in polluted water of tra-fish ponds treatment, reduced TSS, BOD₅, COD concentrations after 72 h and wastewater piggery decreased TSS, BOD₅, COD and TN concentrations, reached to B standard of the **Regulation QCVN 40_2011/BTNMT**. The water after biological treatment with FPB strains can release to canals/rivers or treated with heterotrophic removal bacteria and poly-P bacteria to reach to A standard.

References

1. Ismail MYB. Biological Nutrient Removal Using Sequencing Batch Bioreactor. A thesis of the degree of Bachelor of Chemical Engineering. Faculty of Chemical & Natural Resources Engineering Universiti Malaysia Pahang. 2009.
2. Zhao H, Liu H, Zhou J. Characterization of a bioflocculant MBF-5 by *Klebsiella pneumoniae* and its application in *Acanthamoeba* cysts removal. *Bioresource Technology*. 2013; 137: 226 – 232.
3. Salehizadeh H, Shojaosadati S. Extracellular biopolymeric flocculants: recent trends and biotechnological importance. *Biotechnology Advances*. 2001; 19: 371 – 385.
4. Xia S, Zhang Z, Wang X, Yang M, Chen L, et al. Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. *Bioresource Technology*. 2008; 99: 6520-6527.
5. Qin L, Liu J, Li G, Kang Y. Removal of Tannic Acid by Chitosan and N-Hydroxypropyl Trimethyl Ammonium Chloride Chitosan: Flocculation Mechanism and Performance. *J. Dispersion Science and Technology*. 2015; 36: 695–702.
6. Dearfield KL, Abernathy CO, Ottley MS, Brantner JH, Hayes PF. Acrylamide: its metabolism, developmental and

- reproductive effects, genotoxicity, and carcinogenicity. *Mutat.Res.* 1988; 195: 45–77.
7. Tong Z, Zhe L. Microbial flocculant and its application in environmental protection. *Journal of Environmental Science.* 1999; 11: 1–12.
8. Sponza DT. Extracellular polymer substances and physicochemical properties of flocs in steady and unsteady-state activated sludge systems. *Process Biochemistry.* 2002; 37: 983–998.
9. Yang Z, Yuan B, Huang X, Zhou J, Cai J, et al. Evaluation of the flocculation performance of carboxymethyl chitosan-graft-polyacrylamide, a novel amphoteric chemically bonded composite flocculant. *Water Research.* 2012; 46: 107–114.
10. Yim JH, Kim SJ, Ahn SH, Lee HK. Characterization of a novel bioflocculant, p-KG03, from a marine dinoflagellate, *Gyrodinium impudicum* KG03. *Bioresource Technology.* 2007; 98: 361–367.
11. Gonzalez-Torres A, Putnam J, Jefferson B, Stuetz RM, Henderson RK. Examination of the physical properties of *Microcystis aeruginosa* flocs produced on coagulation with metal salts. *Water Research.* 2014; 60: 197–209.
12. Ugbenyen AM, Cosa S, Mabinya LV, Okoh AI. A. Bioflocculant production by *Bacillus* sp. Gilbert isolated from a marine environment in South Africa. *Applied Biochemistry and Microbiology.* 2014; 50: 49–54.
13. Fattom A, Shilo M. Phormidium J-1 bioflocculant-production and activity. *Archives Microbiology.* 1984; 139: 421–426.
14. Okaiyeto K. Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7. *Int. J. Mol. Sci.* 2015; 16: 12986–13003.
15. Prasertsan P, Dermilim W, Doelle H, Kennedy JF. Screening, characterization and flocculating property of carbohydrate polymer from newly isolated *Enterobacter cloacae* WD7. *Carbohydrate Polymers.* 2006; 66: 289–297.
16. Aljuboori AHR, Uemura Y, Osman NB, Yusup S. Production of a bioflocculant from *Aspergillus niger* using palm oil mill effluent as carbon source. *Bioresources Technology.* 2014; 171: 66–70.

17. Deng SB, Yu G, Ting YP. Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. *Colloid Surfaces B – Biointerfaces*. 2005; 44: 179–186.
18. Kurane R, Takeda K, Suzuki T. Microbial flocculants.1. Screening for and characteristics of microbial flocculants. *Agricultural and Biological Chemistry*. 1986; 50: 2301–2307.
19. Ndikubwimana T, Zeng X, He N, Xiao Z, Xie Y, et al. Microalgae biomass harvesting by bioflocculation-interpretation by classical DLVO theory. *Biochemical Engineering Journal*. 2015; 101: 160–167.
20. Zhang X, Sun J, Liu X, Zhou J. Production and flocculating performance of sludge bioflocculant from biological sludge. *Bioresource Technology*. 2013; 146: 51–56.
21. Hazana R, Norli I, Ibrahimand M, Fazilah A. Flocculating activity of bioflocculant producing bacteria isolated from closed drainage system (CDS) at the Prai Industrial Zone, Penang, Malaysia. *International Conference on Environmental Research and Technology, (ICERT' 08)*. 2008; 422-425
22. Deng SB, Bai RB, Hu XM, Luo Q. Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use in starch wastewater treatment, *Applied Microbiology Biotechnology*. 2003; 60: 588 - 593.
23. Tang J, Qi S, Li Z, An Q, Xie M, et al. Production, purification and application of polysaccharide-based bioflocculant by *Paenibacillus mucilaginosus*. *Carbohydrate Polymers*. 2004; 113: 463–470.
24. Wu JY, Ye HF. Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtilis* DYU1 isolate. *Process Biochemistry*. 2007; 42: 1114-1123.
25. Kurane R, Matsuyama H. Production of a bioflocculant by mixed culture. *Bioscience, Biotechnology, and Biochemistry*. 1994; 58: 1589-1594.
26. Gao J, Bao HY, Xin MX, Liu YX, Li Q, et al. Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31. *Journal of Zhejiang University. Sciences. B*. 2006; 7: 186-192.
27. Kurane R, Hatamochi K, Kakuno T, Kiyohara M, Kawaguchi K, et al. Purification and characterization of

- liquid bioflocculant produced by *Rhodococcus erythropolis*. Bioscience, Biotechnology, and Biochemistry. 1994; 58: 1977-1982.
28. Zhang ZQ, Lin B, Qing XS, Jiang WX, Ming YA. Production and application of a novel bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source. Journal of Environmental Sciences. 2007; 19: 667–673.
 29. Aljuboori AHR, Idris A, Abdullah N, Mohamad R. Production and characterization of a bioflocculant produced by *Aspergillus flavus*. Bioresource Technology. 2013; 27: 489–493.
 30. Lachhwani. P. Studies on Polymeric Bioflocculant Producing Microorganisms. Master Thesis, Thapar Institute of Engineering and Technology. Patiala, India. 2005.
 31. Neumann B, Pospiech A, Schairrer HU. Rapid isolation of genomic DNA from Gram negative. Trends Gent. 1992; 8: 332-333.
 32. Jie G, B Hua-ying, X Ming-xiu, L Yuan-xia, L Qian, et al. Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31. Journal of Zhejiang University Science. 2006; 7: 186-192.