Comparison of Biogas Production Using Swine Manure Derived from Pigs Fed With Probiotics and Tylosin

เปรียบเทียบการผลิตก๊าซชีวภาพจากมูลสุกรที่เลี้ยงด้วยโปรไบโอติกและยาปฏิชีวนะไทโลซิน

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ABSTRACT

Lactobacillus strain KKU-L21 and *Bacillus* strain KKU-B3 were isolated from pig feaces. They possessed probiotic properties including their inhibitory effects against four tested pig pathogens i.e. *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *S. enterica* serovar Choleraesuis and *Escherichia coli* O157:H7. They could well survive the simulated gastric and small intestinal environments which were pepsin solution (pH 2.5) and pancreatin solution supplemented with 0.3% bile (pH 8.0), respectively. These two probiotic bacteria produced organic acids such as acetic, propionic and butyric acids that were necessary for methanogenesis. Biogas production using swine manure derived from pigs fed with probiotics and antibiotic (tylosin) was compared with the one that obtained non-supplemented diets. It was found that the mixed cultures of *Lactobacillus* strain KKU-L21 and *Bacillus* strain KKU-B3 enhanced the production of biogas whereas tylosin displayed inhibitory effects on biogas produced from pig manure.

บทคัดย่อ

แบคทีเรียโปรไบโอติกสายพันธุ์ *Lactobacillus strain* KKU-L21 and *Bacillus* strain KKU-B3 ที่แยกจากมูลสุกรพบว่ามีคุณสมบัติของแบคทีเรียโปรไบโอติกซึ่งประกอบด้วย ความสามารถในการต้านแบคทีเรียก่อโรคในสุกรทั้ง 4 สายพันธุ์ได้แก่ *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *S. enterica* serovar Choleraesuis และ *Escherichia coli* O157:H7 และสามารถรอดชีวิตเมื่ออยู่ในสภาวะจำลองของกรดในกระเพาะ (pepsin pH 2.5) และ สภาวะจำลองของลำไส้เล็กของสุกร (pancreatin ร่วมกับ 0.3 % bile salt, pH 8) นอกจากนี้ทั้งสองสายพันธุ์ยังสามารถสร้างกรดอินทรีย์ที่จำเป็นต่อกระบวนการ methanogenesis ซึ่งได้แก่กรด acetic, propionic และ butyric โดยในการศึกษาครั้งนี้ได้ทดลองเปรียบเทียบการผลิตก๊าซชีวภาพจากมูลสุกรกลุ่มที่เลี้ยงด้วยอาหารผสมแบคทีโปรไบโอติกและอาหารผสมยาปฏิชีวนะไทโลซิน กับสุกรที่เลี้ยงด้วยอาหารปกติ ในการศึกษาครั้งนี้พบว่ามูลสุกรกลุ่มที่ได้รับแบคทีเรียโปรไบโอติกส่งเสริมการผลิตก๊าซชีวภาพ ในขณะที่มูลสุกรจากกลุ่มที่ได้รับไทโลซินส่งให้การผลิตก๊าซชีวภาพลดลงเมื่อเทียบกับกลุ่มควบคุม

Key Words : Biogas, probiotic, antibiotic
คำสำคัญ : ก๊าซชีวภาพ โปรไบโอติก ยาปฏิชีวนะ

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Introduction

The waste from pig farm had increase every year. The large quantity of pig slurry is cause of environmental problems such as increased green house effect, malodours and by direct impact on human health. Anaerobic digestion is the process whereby bacterial break down biodegradable substance to many organic acids until convert organic acids such as acetic, butyric and propionic acids to biogas by methanogenic bacteria. This process is one of cost-effective alternative for wastewater like swine manure. Especially, biogas from anaerobic process it produces renewable energy (methane) and valuable digested residues that can be used as liquid fertilizer and soil conditioner (Bonmatí et. al., 2001; Lier et. al., 2001).

Antibiotics are used widely in many pig farms as feed additives, to prevent pathogens infection and to promote growth at therapeutic level, it is possible that these antibiotics excreted in swine manure may reduce biogas production (Stone et. al., 2009). Many researchers reported some antibiotic such as tylosin, penicillin, lyncomycin and chlortetracycline to reduce biogas production (Masse et. al., 2000; Sanz et. al., 1996; Fischer et. al., 1981). The use of probiotic as alternative to antibiotic has been considered to prevent pathogen and promote growth. The probiotics most commonly used are strains of intestinal origin that belong to the group commonly known as *Lactobacillus* and *Bacillus* (Chang et. al., 2001; Simpson et. al., 2004; Patel et. al., 2009). Organic acid such as, acetic, n- butyric and propionic acids are major end products of microbial break down of carbohydrate in the large intestine. (Macfarlane & Gibson, 1995) Probiotic bacteria have been reported that increase organic acid production in pigs approximately 100 kg when pig fed commercial diet with probiotic bacteria. (Sagata et. al., 2004) Thus, probiotic bacteria supplemented to pig feeds may produce by-product to support methanogenesis and give a positive effect on methane production from swine manure. Therefore, in this study we aimed to examine the effects of probiotic bacteria and antibiotic which present in swine manure on biogas production.

Materials and methods

Bacterial strains

The pig pathogenic strains used as test organisms for inhibition study were *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *S. enterica* serovar Choleraesuis and *Escherichia coli* O157:H7. Each strain was grown on Blood agar prior to suspending in 20% glycerol and kept at -80°C until needed. *Lactobacillus* strains and *Bacillus* strains were isolated from various kinds of samples such as the gastro-intestinal tract of healthy fish, water and sediment around the cultured fish-cages, pig and poultry excrement. *Lactobacillus* strains were cultivated on MRS agar containing 0.5% $CaCO_3$ and incubated at 30°C for 2 days. *Bacillus* strains were cultivated on Nutrient agar and incubated at 35°C for 2 days.

Antimicrobial activity assay

The Antimicrobial activity was detected by the agar well-diffusion assay (Parente et. al., 1995). The *Lactobacillus* spp. were cultivated in MRS broth for 24 h at 30°C without shaking and the *Bacillus* spp. were grown in nutrient broth (NB) on a rotary incubator shaker (150 rpm) at 35°C for 48 h to obtain the culture broth. Test organisms were cultured in Mueller Hilton broth and incubated at 35°C for 18 h. Then, the turbidity of cell suspension was adjusted to an equivalent of McFarland standard 0.5. A sterile cotton swab was submersed into bacterial suspension and the excess fluid was removed prior to evenly swabbing 3 planes onto the surface of Mueller Hilton agar plate. An agar well of 6 mm diameter was punched using a cork borer. A 40 μl aliquot of broth culture of *Lactobacillus* spp. and *Bacillus* spp. were added into an agar well. Incubation was done at 30°C for 18 h. The size of inhibition zones were measured and compared to determine the potentially effective probiotic bacteria.

Simulated gastric and intestinal fluid tolerance test

Tolerance of *Lactobacillus* and *Bacillus* strains to pepsin and pancreatin were studied. Cells of *Lactobacillus* from 24 h-incubation were harvested by centrifugation (5000 x g for 15 min at 4°C) and washed three times with phosphate buffer solution (PBS) pH 7.2. To determine gastric transit tolerance, the washed cells were resuspended in PBS, pH 2.5 containing pepsin (3g/l) and the bacterial suspension was taken at 0, 5, 40 and 180 min of 30°C incubation for total viable cell count. To determine small intestinal transit tolerance, the washed cells were resuspended in PBS, pH 8 containing pancreatin (1g/l) and 0.3 % bile salt and the bacterial suspension was taken at 0, 5, 240 and 360 min of 30°C incubation for total viable cell count (Guerra et. al., 2006; Huang et. al., 2004).

For *Bacillus* strain, cell suspension from 36 h-incubation was heated at 80°C in water bath for 15 min prior to centrifugation (5000 x g for 15 min at 4°C) and washed twice with phosphate buffer solution (PBS) pH 7.2. The washed spores were resuspended in simulated gastric and intestinal fluid as described earlier for *Lactobacillus*. Viable cell counts of *Bacillus* in simulated gastric fluid were determined at 0, 5, 40 and 180 min of 30°C incubation whereas the counts were done at 0, 1, 2, 3, 4 and 5 day of incubation for intestinal tolerance (Hyronimus et. al., 2000; Spinosa et.al., 2000).

Production of organic acids from probiotic bacteria

Bacillus strains were cultivated in Glucose-Yeast extract-Peptone broth (GYP broth: 2% glucose, 0.5 % yeast extract, 0.5 % peptone) and incubated at 35°C for 24 hours. *Lactobacillus* strains were grown in MRS broth and incubated at 30°C for 24 hours. The culture broth was centrifuged at 5000 x g for 15 min and the obtaining supernatant was then filtered through 0.45 μm membrane and kept frozen until needed. Organic acids (acetic, butyric, formic, and lactic acids) were determined by reverse phase HPLC (Waters 600 series, USA) using C-18 column (Prevail organic acids, 4.6 x 250 mm). A solution of 25 mM KH_2PO_4 (pH 2.5) was used as mobile phase at a flow rate of 1.0 ml/min and components were measured by UV detector at 220 nm-wavelenght.

Animal medication and biogas production in serum bottle

Twelve pigs (3 months old), ranging from 70 to 90 kg in body mass, were kept in pens. Pens were scraped clean daily. Pig feed contained a mixture of 7% poultry meal, 3.5% fish meal, 7% soybean meal, 15% defatted rice bran, 38% ground corn, 0.2% lysine, 0.05% methionine, 0.5% P21, 0.3% salt, 0.5% premix and 15% biomass.

Pigs were assigned randomly to groups i.e. control fed with pig feed, probiotics (fed with pig feed supplemented with probiotics KKU-L21 and KKU-B3 at 10^7 CFU/g each) and antibiotic group (fed with pig feed supplemented with tylosin 150 mg/kg).

After one week-acclimatized period for the pigs, manure from each pen was collected for anaerobic fermentation in serum bottles for biogas production using the sludge inoculum obtained from an anaerobic digester of the private swine husbandry in Khon Kaen province. The volume of biogas produced in serum bottle was measured daily by the water displacement method (Varel et. al., 1997).

Results and discussion

Screening of potential probiotic bacteria: *Lactobacillus* and *Bacillus* strains

From the isolation processes, fifty bacterial strains showed inhibitory effects on *Salmonella enterica* Enteritidis, *S.* Typhimurium, *S.* Choleraesuis and *Escherichia coli* O157:H7. Some of these results are shown in Table 1. Identification of the bacterial isolates by conventional methods revealed that thirty-five isolates were belonged to a genus *Lactobacillus* and fifteen isolates were belonged to a genus *Bacillus.*

The effects of simulated gastric and intestinal fluid on the viability of the probiotic *Lactobacillus* and *Bacillus* strains are shown in Fig. 1 and Fig. 2 respectively.

It was found that after 3 h-incubation in simulated gastric and intestinal fluids, *Bacillus* strains tolerated the harsh conditions better than *Lactobacillus* did. Subsequently, the most tolerant strains, *Bacillus* KKU-B3 and *Lactobacillus* KKU-L21 were chosen to be used as mixed bacterial probiotics. There had been reports on using mixed culture of bacteria that exhibited satisfactory effects as probiotics (De Angelis et. al., 2006; Guerra et. al., 2006)

Table 1 Inhibition zones generated by potentially probiotic bacteria when incubated with four bacterial pathogens

Isolates	Diameter of inhibition zone (mm)			
	***S.* Enteritidis**	***S.* Choleraesuis**	***S.* Typhimurium**	***E. coli* O157:H7**
L87A	10	10	10	16
L87B	10	9	11	15
KKU-L21	19	18	17	12
L8014	11	10	12	11
L53	8	8	9	7
L389	11	10	10	10
KKU-B2	NI	13	13	13
KKU-B3	NI	13	12	10
KKU-B22	NI	15	14	19

NB; NI, not inhibition

Organic acids production by probiotic strains

Certain organic acids are important intermediates in methanogenesis. Fifty strains of the potential probiotic bacteria were investigated for their organic acids production. It was shown that *Lactobacillus* strains produced acetic, butyric, lactic and formic acids whereas *Bacillus* strains produced only butyric acid. Some of these results are shown in Table 2.

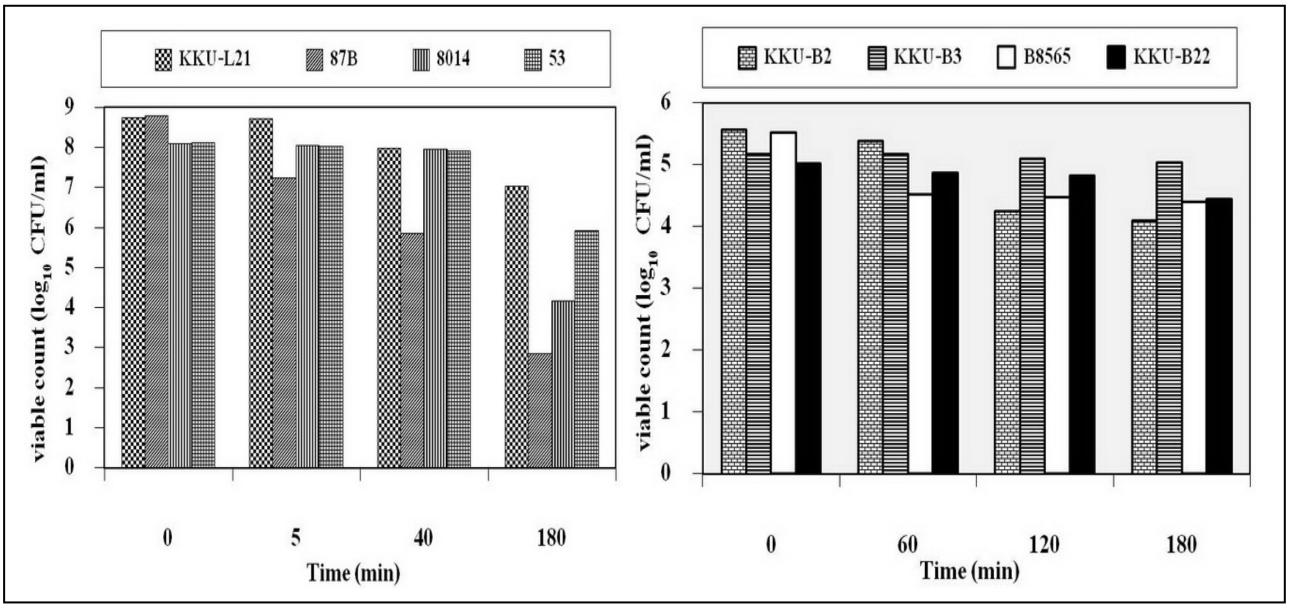

Figure 1 Effect of simulated gastric fluid on viability of *Lactobacillus* (a) and *Bacillus* (b) strains.

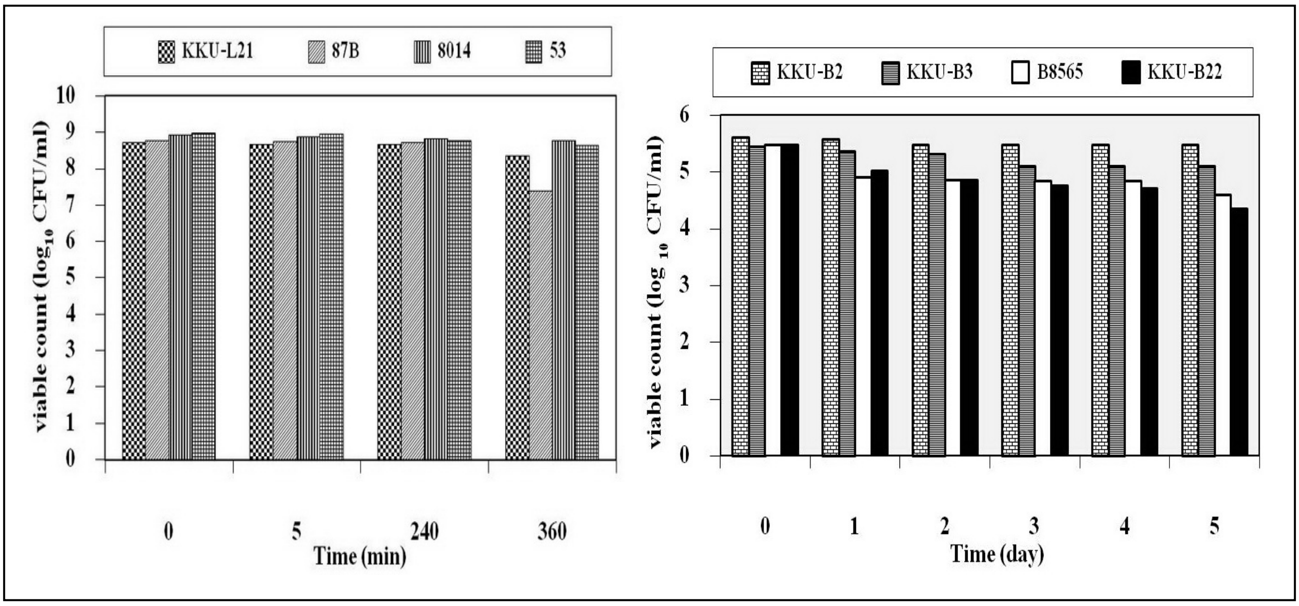

Figure 2 Effect of simulated intestinal fluid on viability of *Lactobacillus* (a) and *Bacillus* (b) strains.

Table 2 Organic acids production from probiotic bacteria

isolates	Organic acid production (g/l)			
	Acetic	Butyric	Formic	L-lactic
KKU-L21	5.52	47.93	16.39	13.34
L87B	6.0	41.67	15.44	6.21
L8014	5.52	17.44	15.06	14.58
L53	5.88	25.90	15.61	9.12
KKU-B2	-	6.78	-	-
KKU-B3	-	6.52	-	-
KKU-B22	-	6.60	-	-
B8565	-	7.22	-	-

Some criteria to be considered in selecting probiotic bacteria for application in pigs are according to their antimicrobial activities against pig pathogens, tolerance to gastric and intestinal fluids, and ability to produce high amount of volatile fatty acids. (Bogovic et. al., 2004; Lahteinen et. al., 2009). Therefore, *Lactobacillus* strain KKU-L21 and *Bacillus* strain KKU- B3 were chosen for feeding experiments on the rationale that if they could survive through gastrointestinal tract of pig, the high number of these strains in excrement might support the anaerobic digestion of pig manure and enhance the biogas production.

Biogas production in serum bottles

Biogas production using manure from three groups of experimental pigs (control, probiotics, antibiotic) were monitored daily for 2 weeks. The results from this study are shown in Fig. 3.

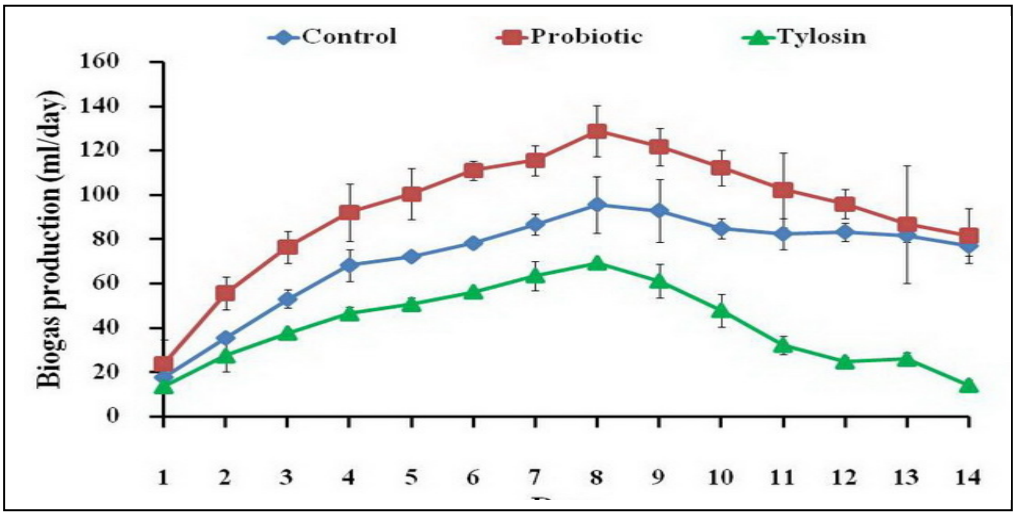

Figure 3 Biogas production from pig manure contain probiotics and tylosin

It was found that all three types of pig manure produced highest biogas in 8 days. The highest volume of biogas obtained from the control, probiotics and tylosin group were 95.80, 128.84 and 69.37 ml/day, respectively. The composition of methane in biogas in all experiments were greater than 70% indicating the good quality of biogas produced. Although the percentages of methane in biogas in all three experimental groups were similar but there were obvious differences in biogas volume produced.

It was clearly shown that the biogas production from antibiotic (tylosin)-treated group was 27.59% lower than the control group. Interestingly, the biogas production from probiotic-treated group was 34.44 % higher than the control group.

Stone et. al. (2009) reported that tylosin which remained in pig excrement decreased biogas production. Our finding results were also consistent with the ones described by Masse et. Al. (2000) and Loftin et. al. (2005) that tylosin had inhibitory effects on methane production in anaerobic digestion.

Conclusions

In this study, two effective strains of probiotic bacteria were obtained i.e. *Lactobacillus* KKU-L21 and *Bacillus* KKU-B3. Supplementing these two bacterial strains into pig diets clearly enhanced biogas production from pig manure whereas manure from the pig fed with antibiotic, tylosin, showed negative effects on anaerobic digestion and deceased biogas production when compared with the manure from control group that derived from pigs fed with non-supplemented diets.

Acknowledgements

This research has been financially supported by the Research Center of Agricultural Biotechnology for Sustainable Economy and Center for Agricultural Biotechnology, (Khon Kaen University) under the Project for Graduate Study and Research in Science and Technology and The Khon Kaen university's graduate research fund academic year 2009. The authors would like to thank Mr. Wanchai Hinthong at Premer Co. Ltd. for a valuable discussion.

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