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Exploration of Natural Probiotics From Pineapple Peels (Ananas Comosus) as a Source of Feed Supplements for Ruminants

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Abstract
The Aims of this research was conducted to evaluate, isolate and identificate of the pineapple peel as source of probiotics bacteria and identified of potentially to be used as supplement for feed of ruminant. The research were divided into three stages. The first stage were done to identification of nutrient and phytochemical contens of pineapple peel, the second stage were to isolation and identification of microbial bacteria from pineapple peel. The third stage were done carried out of the best carrier for microbial life (rice brand, crown, leaf and pineapple peel). Bacterial isolation was performed according to Hadioetomo method (1993). Identification of gene sequence were done PCR. The results ofthe research that in the first stage nutrient content of the pineapple peel there were 3.85% ash; 27.09% crude fiber; 1.15% crude fat; and 8.78% crude protein; 59.13% BETN. The Pineapples peel contained of phytochemicals there were 9 mg beta-carotene, alkoloid, flavonoids, triterpenoids, and phenolic. The results of the second stage of this research show that of the isolation of bacteria presented in the peel of pineapple had five bacterial isolates. The five bacterial isolates were than identified by DNA extracting with conducted molecular PCR. The results squencing showed that the bacterial of Stenotrophomonas maltophilia strain W8-1 16S rRNA, Bacillus pumilus strain L 1 16S rRNA, Stenotrophomonas maltophilia strain Dh 16S rRNA, Stenotrophomonas maltophilia strain PBBC9 16S rRNA, Stenotrophomonas maltophilia strain PBBC9 16S rRNA. Fifth isolates selected one of them as a potential bacteria as probiotic bacteria is both the bacterial isolate Bacillus Pumilus strain L 1 16S rRNA. The Conclusion that the experiment carried out of Bacillus Pumilus bacteria strain L 1 16S rRNA were suitable to growing in the pH of 6 - 7.5, temperature of 37-50^{o}C and in the carrier of rice bran. Bacillus Pumilus bacteria strain L 1 16S rRNA gene to be used feed supplements for ruminant.
Keywords : Exploration, pineapple peel, probiotic, Supplement

1. Introduction

Indonesia ranked sixth highest pineapple producer after Thailand, Brazil, Costa Rica, Philippines and China (FAOSTAT, 2011; Mulyono et al., 2013). Jambi provinces have large of product of pineapple peel as waste of agro-industry by product. The central of statistics of Jambi Provinces Goverment reported that the number of pineapple production as by product in Jambi were rich at 74,816 tons a year, and the highest production centre was in Muaro Jambi district, at the Tangkit village with production were rich at 74,232 tons a year (BPS Jambi, 2010). This means that the availability of pineapple peel in Jambi Province is large enough with (about 2,9926.4 tons a year or 40% from the production of pineapple). If the waste were not used, they can give some problems such as environmental pollution. On the other hand, the used of pineapple as an alternative feed for ruminants as one of solution.

Pineapple (Ananas comosus) is one of the crops grown in Indonesia, including the types of pineapple are *Cayenne* or *Queen*. Prospects agribusiness of pineapple in the future are very good with the tendency to increase both for fresh fruit and processed materials such as lunk head as pineapple, fried pineapple, pineapple syrup etc. Pineapples are rich in potassium, calcium, iodine, Sulfur, Chlorine, Acid, Biotin, Vitamin B12, Vitamin E and Bromelain enzymes. The productions of pineapple waste as by products are approximately 75 - 85% of the production of pineapple peel, crown and core, while the pineapple peel alone is for about 30 - 35% (Lubis, 1991).

Wijana et al., (1991) reported that the pineapple peel contains 81.72% water; 20.87% crude fiber; 17.53% carbohydrate; 4.41% and 13.65% protein and reducing sugar. Pineapple peel also contains 8.01% crude protein; 0.95% crude fat; crude fiber 20.84%; 13.52% and 4405 Kkal/kg GE (Budiansyah et al, 1999). Based on the content of nutrients, the pineapple fruit contains high enough carbohydrates and sugar. Those, it could be medium for the bacteria to grow in the fruits. The kinds of bacteria whose can grow in that fruits as medium should be detected and isolated.

Utilization of pineapple peel as animal feed was constrained by the quality with a very low protein content of 5.88 % and a high crude fiber (13.16 %). It is necessary to adding on the pineapple peel such as probiotics or others to increase of its digestibility. According to Hanafi (2004) reported that in agricultural wastes, such as bark of pineapple had properties in low nutritional value, especially proteins content and low digestiblity, generally. Its often had any substance or component like as less anti nutritional that caused toxicity on the animal or potentially on pollution.

One of some effort to increase the nutritional value of local waste as animal feed example pineapple peel

could be done of applied technology by processing with the addition of bacterial as probiotics or using the chemicals substance. Preston and Leng (1987) reported that there are some processing to improve the digestibility of crude fiber from low-quality feed ingredients either through chemical processes, physical and biological processes. Handling of agricultural waste by biological process could be done by using the enzyme cellulase, or probiotics supplementation. But all of there method had not yet optimum result to the productivity of the animal. Therefore, to optimize bioprocess the research were done by addition or supplementation of probioticsin diet by formulation of feed for Ruminants.

The use of probiotics were used widely in the world of farming and also aims to avoid harmful side effects on antibiotic using. It can not be separated from the efforts to give the nutritional requirement for life of rumen microbial and increased efficiency of yield of microbial protein synthesis in the rumen, and at the end could be increase the productivity of animal livestock (Winugroho, 1995). The probiotics bacteria were searched from with isolation and identification.

2. Reseacrh Methode

The research were conducted at the Laboratory of Ruminant Nutrition Faculty of Animal Husbandry, Jambi University for proximate analysis, chemical laboratory of Andalas University for phytochemicals analysis, laboratory of BPVT, Bukittinggi for Isolation of bacteria and searching for the best carrier for microbial life, and Biotecnology laboratory LIPI, Bogor for identification gene using PCR instrument. The research were done into 3 (three) stages, first stage was to exploration of pineapple peel by analyzing the phytochemical content in the peel of pineapple. The second stage the research were to isolation and identification of bacteria in the pineapple peel that was potentially as probiotic. The third stage was to find out of the best carrier bacteri for microbial life by searching the bacteria using incubation of bacteria in some of the medium incubation.

Parameters of the experiment :

1) Eksploration of Pineapple Peel. Exploration of food substances in pineapple peel product must be known by proximate analysis (AOAC,1980). The proximate analyze were done to know the nutrition content such as, crude protein, crude fat, crude fiber and nitrogen free extracts in the pineapple peel.
2) Isolation and Identification. Isolation of bacteria includes several activities, among others : dilution at planting, incubation, purification, enrichment, and storage (Hadioetomo, 1983) .
   The pineapple peel as material of this research were obtained from by product of household industry. Medium incubation for bacteria were used : nutrient agar (NA), MRSB, BPW, aquades, spritus. Some of the tools were used in the experiment such as vortex, scala glass, erlemeyer, micro pipette, measuring cups, petridisc, test tubes, autoclave, oven incubation, aluminar flow, electric heating, colony counter.
   Bacteria were isolated by the method, 5 grams of pineapple peel were taken and grown on media MRSB, then diluted with the series up to 10^5 and then bacteria was grown on medium of NA (Nutrien Agar) and incubated for 24 hours. The number of colonies of bacteria that was grow in the media then were counted by colony counter machine. The morphology of bacteria were identification by colouring or planting reaction, and dominan bacteria form colouring or planting were selected of bacteria. Identification of bacteria then were continued by extracting DNA genome using PCR electrophoresis and scequencing.
3) Choosing The Best Carrier for Microbial Life. The research also were done to find out the carrier medium that was suitable for bacteria life. The research were used some of component pineapple that were pineapple peel, pineapple crown and leaf and raw of the carrier medium life. The best results in cultivation of bacteria that was marked by in higher number of colony bacteria that were grown and longer life ability in storage.

3. Results And Discussion

Nutrien Content of Pineapple Peel

Proximate analysis nutrien content of pineapple peel showed that the crude protein content was higher than in the previous studied reported by Mangunwijaya et al., (2012), Sidharta (1989) and Hartadi et al., (1989) (Table 1). Based on the nutritional content, the pineapple peel still be used as foodstuffs for ruminants.When the peel pineapple was used as much as 40% in the diet, the value of TDN was higtest while the peel in combination with grass and calliandra as much as 40% in sheep diet.

The pineapple peel contain phytochemical compounds.

The constituents of phytochemical in the peel of pineapple that were found 9 mg beta-carotene, alkoloid, flavonoids, triterpenoids, phenolic. Beta-Carotene/β-Carotene is anorganic compound and classified as aterpenoid. β-Carotene is a pigment in red-orange which tends to be highly colored and contained in large quantities of plants and fruits including pineapple peel. Phytochemical compounds can optimize the fermentation pattern in the rumen by microbes. The use of pineapple peel up to the level of 15% in the diet of rabbits will not lower the and digestibility of dry matter and organic matter (Ningrum, 2010). The presence of phytochemical compounds found in the pineapple peel shows function as animal feed may give importance mainly for the

health of livestock and livestock products making use of leather as a material pineapple concentrate food which is expected to increase livestock productivity and product quality.

Isolationand Identification of Bacteriaon Pineapple Peel

The research were isolated the microbes on the Peel of fresh pineapple, it were found five isolates of bacteria gram positive dominant. Five isolates of pineapple peel were looked at in Figures 1 and 2. Five bacterial isolates were identified by extracting DNA genome conducted molecular PCR. Partial results squence (NCBI BLAST) were : the first isolat was Stenotrophomonas maltophilia strain W8-1 16S, 2nd isolat was Bacillus pumilus strain L1 16S rRNA, 3nd isolat was Stenotrophomonas maltophilia strain Dh 16S, 4nd isolate was Stenotrophomonas maltophilia strain PBBC9, isolat 5. Stenotrophomonas maltophilia strain PBBC9 16S. Form five of the isolates were selected one of them as a potential bacteria as probiotic bacteria is both the bacterial isolate Bacillus Pumilus strain L1 16S rRNA. Results idenification of pineapple peel isolates can be seen in Table 2.

Form figures 1 and 2 and table showed that the bacteria in use or being used as inoculum for a feed supplement in the fourth stage of the research would be applied to ruminants was a bacterium of Bacillus pumilus strain L1 16S rRNA. Bacillus pumilus strain L1 16S rRNA was classified as genus Bacillus sp which is a gram-positive, rod-shaped, can grow in aerobic and anaerobic spores are resistant to heat (high temperature), capable of degrading xylan and carbohydrates (Cowan and Steel's, 1973). Bacillus pumilus based on morphologic classified by the type of bacillus spores spongarium elipsoidal with no swelling (Slepeckly in Doi and Mcgloughlin, 1992 in Hatmanti, 2000). Bacterium Bacillus pumilus sourced from soil, water, air and plant roots in some decomposed (Cowan and Steel's, 1973). Pumilus bacteriais division or group of Bacillus sp bacteria. Several Bacillus species produce extracellular enzymes such as protease, lipase, amylase, and cellulase which can aid digestion in animals (Wongsa and Werukhamkul, 2007). Antibiotic compounds which were produced by Bacillus sp as like as bacitracin, pumulin, laterosporin, gramisidin, and tirocidin effective against. Gram-positive bacteria as well as colistin and polymyxin are effective against gram-negative bacteria or bacterial pathogens. Some species of Bacillus sp. which produce antibiotics can be used as biological agents. Some types of antibiotics were produced of them that were in form of iturin, surfactin, fengicin, polymyxin, difficidin, subtiline, and mycobacilin , while difficidin have a wide spectrum, mikobacilin and zwittermicin are anti fungal (Todar, 2005).

All of five isolates were identified by PCR procedure, one of them were selected that was 2nd isolate (Bacillus pumilus strain L1 16S rRNA) because based on previous reported showed that the bacteria Bacillus pumilus strain 16S rRNA gene L1 is being including probiotic bacteria potentially, and it was used in ruminant diet as feed supplement. Some types of the Bacillus (B. Cereus; B. clausii and B. pumilus) is being including in the five commercial probiotic products until now. The were consist of bacterial spores that have been characterized and very potential for colonization, immunostimulasi, and antimicrobial activity (Duc et al., 2004).

One alternative to reduce the problems of feed and animal nutrition were using of probiotics as feed supplement or growth promotor. Using of some growth promoters like probiotics and prebiotics which have positive effect on animal's growth performance (Vandana at al., 2013). Substance of toxic compounds whice were produced in the metabolism of probiotic bacteria such as lactic acid, hydrogen peroxide, bacteriocins that are antimicrobial and antibiotic could be able to reduce the growth of pathogenic bacteria (Yulinery et al., 2006). Form of bacterial pumilus strain L1 16S rRNA were obtained in this experiment could be seen in Figure 3.

Based on Figure 3, the bacteria pumilus strain 16S rRNA gene L1 was appeared rod-shaped and white colony color. The type of Bacillus sp. Showed of different form colonies on agar. Colony shape and size of the bacteria were varied and depended on the type. In addition, each type also shows the ability and resistence in the the different environmental conditions, such as resistance to heat, acid solution, salt content, and so on (Rheinheimer, 1980). The *B. pumilus* isolates were further characterized by arbitrarily primed PCR (AP-PCR), antibiotic sensitivity profile and PCR screening to know toxin genes associated with *Bacillus* spp (Ammini at al., 2009).

So that, if we will use the bacteria in animal feed, it must be grown on a suitable carrier material and could be stored in a long time. Bacterium *Bacillus pumilus* strain L1 16S were isolated from pineapple waste to choose one of all part of pineapple plant such as pineapple peel, pineapple leaf and pineapple crown and the rice bran was as the control, it was shown in Table 2.

Generally, bacteria required energy and other materials to build their cells for synthesis their protoplasma or other parts of cell. Bacteria were very dependent on the supply of exogen substances (which comes from outside of the body) for growing, thrive and survive, then the nutrient carrier must contain elements of energy sources, carbon, nitrogen and other inorganic elements, organic molecules, complexes, acid - a fatty acid acids - amino acids, and vitamins.

Nutrient or food should provide enough energy to maintain body functions, activities and growth of living things, such as water, energy sources, carbon source, nitrogen source, sulfur source, a source of phosphorus, a source of oxygen, a source of electron acceptors, mineral resources, and growth factors (Haribi and Ratih, 2008).

Based on Table 2. showed that the rice bran was the best of carrier material to be applied on livestock because the storage time of of the bacterian in that material could be longer than other material, so until 21 days the bacteria can grow well. Rice bran has enough potential to be used as source of energy in ruminant ration and its microbes the rumen, in other hand the rice bran was suittable for the bacteria *Bacillus pumilus*. From Table 2. Nutritional content of rice bran is the number one quality factory I 1.9 % crude protein; metabolizable energy in 2200 kkal/kg; fat 12,l %; 10.0 % crude fiber; phosphorus 1.3 % 0; calcium 0.1 % (Hartadi et al., 1993). It showed that the nutrient content of the bran capable to supply of nutrients for needing to microbial life including bacteriaof *Bacillus pumillus* strain L1 16S rRNA. The using of additives in the manufacture of raw silage with different levels with the addition of Lactobacillus plantarum bran I BL - 2106 cfir/g forage gived effect on some parameters quality silage (Ridwan et al., 2005). *Bacilluspumillus* bacteria strains L1 16S rRNA were growed well when it planted in the rice bran compared in other material of pineapple plant. This bacteria is a probiotic supplement that will be applied in the goat fodder in future studies. So that probiotics could be used as an animal feed supplement, but it must be growth and alive in the rumen conditions, it can be seen in Table 3.

From Table 3. the bacterium *Bacillus pumillus*strains L1 16S rRNA was thermophilic bacteria (37-50^{0}C) and it was more resistance to acids and alkalis (pH 6 - 7.5) and had the ability of live the same as with probiotics bacteria. In this study, the bacteria of *Bacillus pumillus* strain L1 16S rRNA showed be able to live in rumen conditions. *Bacillus pumilus* is including bacteria a spore-forming bacteria, rod-shaped, Gram-positive, and aerobic. It found in soils and some colonize in the root area of some plants, where *B. pumilus* has antibacterial and antifungal activity, and the cellular features of *B. pumilus* are synonymous with other species of the genus Bacillus including *B. subtilis*, *B. megaterium*, and *B. cereus*. (NCBI, 2008). Bacillus sp. also have evolved so that they can live under conditions although harder and faster to obtain protection against stress situations such as low of pH (acidic), alkaline, osmotic, or oxidative conditions, and heat or ethanol. Bacteria ethanol has only one DNA molecule that contains a set of chromosomes, that were some of it's advantages. According Kanmani et al., (2010) one of the characteristics of probiotic bacteria that was had a high ability resistance to acids.

4. Conclusion

The result of this study concluded that :

a. Pineapple peels containing phytochemical component such as alkoloid, flavonoids, triterpenoids, phenolic and 0.09% beta-carotene.
b. Five strain of microbial were founded in the pineapple peels such as *Stenotrophomonas maltophilia* strain W8-1 16S ribosomal RNA, *Bacillus pumilus* strain L 1 16S ribosomal RNA, *Stenotrophomonas maltophilia* strain Dh 16S ribosomal RNA, *Stenotrophomonas maltophilia* strain PBBC9 16S ribosomal RNA, *Stenotrophomonas maltophilia* strain PBBC9 16S ribosomal RNA gene, *Stenotrophomonas maltophilia* strain W8-1 16S ribosomal RNA gene.
c. *Bacillus pumilus* strain L 1 16S ribosomal RNA in the pineapple were indentified as probiotics microbial.
d. *Bacillus pumilus* strain L 1 16S ribosomal RNA were suitable to growat pH 6-7.5, temperature at 37-50^{o}C and rice bran was a suitable carrier material. The *Bacillus Pumilus* bacteria strain L 1 16S rRNA gene was to be used as feed supplements in the diets for ruminant

Reverence

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Table 1.Proximate Analysis Results of Pineapple Peel

Parameters	**Results (%)**	**Previous studies (%)**		
		a	**b**	**c**
Ash	3.85	3.51	3.80	4.20
Crude Fiber	27.09	33.25	15.80	14.20
Crude Protein	8.78	4.93	4.50	3.30
Crude Fat	1.15	1.82	1.60	1.70
Non NFE (BETN)	59.13	60.00	78.10	80.00

(a). Mangunwidjaja et al., (2012)
(b) Sidharta (1989)
(c) Hartadi *et al.* (1989)

Table 2. Results of the NCBI Blast Isolate of Pineapple Peel

No	ID	Blast Search NCBI Result	Query Lengt	Homology Y (%)
1	Peel pineapple 1	Stenotropomonas maltophilia strain W8-1 16S ribosomal RNA gene, partial sequence	1418	99
2	Peel pineapple 2	Bacillus pumilus strain L1 16S ribosomal RNA gene, partial sequence	1430	99
3	Peel pineapple 3	Stenotropomonas maltophilia strain Dh 16S ribosomal RNA gene, partial sequence	1391	99
4	Peel pineapple 4	Stenotropomonas maltophilia strain PBCC9 16S ribosomal RNA gene, partial sequence	1421	99
5	Peel pineapple 5	Stenotropomonas maltophilia strain PBCC9 16S ribosomal RNA gene, partial sequence	1435	99

Table 3. Viability of Bacteria In Temperature and pH (CFU/ml)

pH	Temperatures (^{0}C)		
	37	40	50
6	130×10^{15}	250×10^{15}	110×10^{20}
6.5	100×10^{25}	30×10^{25}	270×10^{20}
7	40×10^{25}	30×10^{25}	51×10^{21}
7.5	100×10^{24}	100×10^{24}	290×10^{20}

Table 4. Bacteria Test Matches In Carriers and Storage Long

Storage (day)	Carier	Code	Dilution	Keterangan
			10^{-5} 10^{-6} 10^{-7}	
2	Crown pineapple	M.2.1		Not grwoth
		M.2.2		Not grwoth
	Peel pineapple	K.2.1	10-7	growth 1 coloni
		K.2.2		Not grwoth
	Leaf pineapple	Dn.2.1		Not grwoth
		Dn.2.2		Not grwoth
	Rice bran	Dd.2.1		Growth ¼ plate coloni
		Dd.2.2		Full growth 1 plate
7	Crown pineapple	M.7.1		Not growoth
		M.7.2		Full grwoth 1 plate
	Peel pineapple	K.7.1		growth ½ plate
		K.7.2		growth ¾ plate
	Leaf pineapple	Dn.7.1		Full growth 1 plate
		Dn.7.2		Full growth 1 plate
	Rice bran	Dd.7.1		Full growth 1 plate
		Dd.7.2		Full growth 1 plate
14	Crown pineapple	M.14.1		Growth ½ plate
		M.14.2	10^{-6}	Not growth
	Peel pineapple	K.14.1		Full growth ½ plate
		K.14.2	10^{-6}	Nothing growth
	Leaf pineapple	Dn.14.1		Growth ½ plate
		Dn.14.2	10^{-6}	Growth is coloni 32
	Rice bran	Dd.14.1		Full growth 1 plate
		Dd.14.2	10^{-6}	Full growth 1 plate
21	Crown pineapple	M.21.1	10^{-5} 10^{-6}	Not growth, 10^{-7}growing
		M.21.2	10^{-7}	Not growth
	Peel pineapple	K.21.1		Not growth
		K.21.2	10^{-5}	50 koloni, dilution 10^{-6} 10^{-7}not growth
	Leaf pineapple	Dn.21.1	10^{-7}	Growth
		Dn.21.2		Nothing growth
	Rice bran	Dd.21.1		Full growth 1 plate
		Dd.21.2		Full growth 1 plate

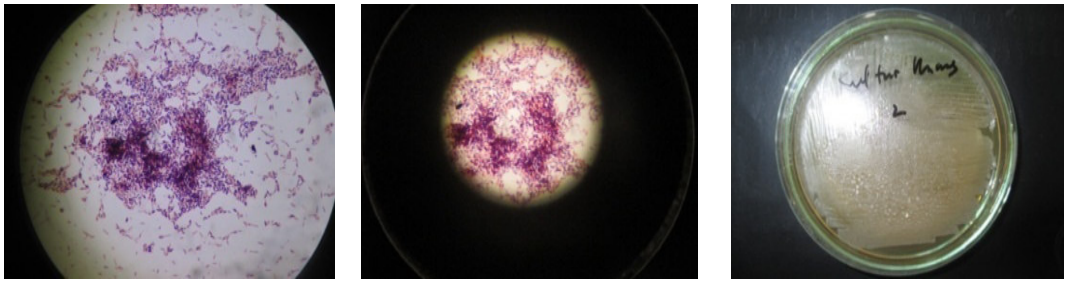

Figure 3.
Bacteria *Bacillus pumilus* strain L1 rRNA from Pineapple peel

Figure 4
isolates isolated to be identified

Figure 5
isolates to be stored