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Isolation and Identification of Epiphytic Lactic Acid Bacteria from Guinea Grass (*Panicum maximum*)

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Abstract: Problem statement: Bacteria can perform a variety of beneficial functions, for example many lactic acid bacteria are responsible for fermentation of silage in the process of forage conservation. In the making of silage, epiphytic lactic acid bacteria are usually insufficient in numbers to promote efficient lactate fermentation. This study was conducted to identify the predominant indigenous bacteria, with emphasis on lactic acid bacteria, from Guinea grass (*Panicum maximum*). **Approach:** Two different condition of growth using nutrient and MRS agar were prepared for isolation of the bacteria. In total, 18 purified isolates were identified by BIOLOG identification system which comprised of 9 bacterial species. Standard plate count in the both conditions was considered. **Results:** Three bacterial species based on the first condition of growth were identified which were belonging to *Flavimonas oryzihabitans*, *Enerobacter cloacae*, *Sphingomonas paucimobilis* B. Lactic acid bacteria based on the second condition of growth were belonging to *Weissella confusa*, *Weissella paramesenteroides*, *Leuconostoc mesenteroides* ssp. *dextranicum*, *Lactococcus lactis* ssp. *hordniae*. Result of plate count showed that 8.3×10^3 CFU lactic acid bacteria are available per gram of fresh guinea grass. **Conclusion:** Three hetero-fermentative and one homo-fermentative lactic acid bacteria were identified which would be suggested to use as bacterial inoculants because of the insufficient amount of epiphytic lactic acid bacteria and the availability of pathogenic bacteria in the grass.

Key words: Isolation, identification, predominant bacteria, guinea grass

INTRODUCTION

The natural feeds for ruminants are generally grasses, forbs and forages. Grassland either native or improved, are predominantly used in ruminant production systems throughout the world. Guinea grass (*Panicum maximum*) is one of the grass species of tribe Paniceae under the Gramineae family (Mcdonald *et al*., 2002). This grass is a native of Africa which has warm climate with over 900 mm rainfall (Barnes *et al*., 2007).

Lactic acid bacteria are involved in the fermentation of silage and lower the pH of the grass to about 4 (Stiles, 1996) whereas other unwanted bacteria such as Clostridia or Enterobacteria increase the pH value, with the acetic acid and butyric acid in silage and hence producing ammonia-N, amines, keto acids and fatty acids and decreasing the nutritional value (McDonald, 1981). Bacterial inoculants can speed the process of lactic acid fermentation and improve preservation of forage because epiphytic LAB is often insufficient in numbers for efficient lactate fermentation (McDonald, 1981). For example, Bureenok showed *et al*. (2005) that increasing the volume of fermented juice of epiphytic lactic acid bacteria as inoculants resulted in increased production of high fermentative quality in guinea grass silage.

MATERIALS AND METHODS

Guinea grass (*Panicum maximum* var. trichoglume) was planted at the University Putra Malaysia's farm in a size of approximately one hectare. Samples of whole plant at about 5 weeks re-growth were taken randomly (Mislevy, 1985) and cut into pieces of about 1 cm by using sterile scissors. Isolation of both predominant indigenous bacteria and epiphytic lactic acid bacteria was carried out in two different methods. For predominant indigenous bacteria, 10 g sample of the fresh grass was homogenized for 2 min in 90 mL sterile peptone water (25.5 g L^{-1}), using a vortex. Ten-fold serial dilution (10^{-1} till 10^{-5}) was then carried out in

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triplicates and 100 µL of last dilution was placed on nutrient agar (20 g L^{-1}) (Merck, Germany) and incubated at 35°C under aerobic condition until colonies were visible. For epiphytic lactic acid bacteria, 10 g sample of the grass was homogenized for 2 min in 90 mL sterile MRS broth (52.5 g L^{-1}) (Merck, Germany), using a vortex. 10-fold serial dilution (10^{-1} till 10^{-3}) was then carried out and kept in an incubator at 30°C for 3 h. 100 mL of last dilution was then placed on MRS agar (68.2 g L^{-1}) (Merck, Germany) in triplicates and kept in anaerobic condition using anaerobic jar. Finally, they were incubated at 30°C until colonies were visible. Standard plate count in both methods was done in triplicates.

Single colonies from both methods based on morphology were chosen by using a bright-field microscope (Nikon, E200. USA). Then, they were sub-cultured for two or three times to get purified bacteria. The gram staining test using the four reagents (crystal violet, acetone, safranin and iodine) were conducted. Secondary staining test, KOH test, was considered as a confirmation for gram staining. Later, Oxidize test were done for the gram negative isolates to determine whether they are enteric or non enteric microbe.

Identification of the isolates was carried out by using BIOLOG identification system. Fresh purified culture of the isolates were inoculated into Gram Negative/Positive (GN/GP) inoculation fluid for indigenous isolates and Anaerobic (AN) inoculation fluid for the isolates of lactic acid bacteria by using sterile cotton swap. Turbidity of the inoculants was adjusted to 63% for enteric bacteria and lactic acid bacteria isolates and 52% for non enteric bacteria by using turbidity meter. Isolation of gram negative enteric bacteria required the addition of thioglycolate (3 drops) as an anti capsulate agent which reduced production of bacterial capsules and hence, strains gave more consistent patterns. Following this, gram negative and anaerobic micro plates were prepared and kept in aerobic and anaerobic condition respectively, for 4-24 h. Finally, reading of these micro plates were carried out using BIOLOG reader (Micro log system™, Release 4.0).

RESULTS

A total of 18 isolates were obtained from the two methods of isolation. Results of gram staining test, KOH test and oxidize test (for gram negative bacteria) are shown in Table 1. Indigenous isolates were gram negative and oxidize positive except of isolate number 1-1 which was gram negative and oxidize negative.

Table 1: Result of gram staining test, KOH test and oxidize test and morphology of all the isolates

Isolates	Gram staining test	KOH test	Oxidize test	Shape
*1-1	-	-	-	Rod
1-2	-	-	+	Rod
1-3	-	-	+	Rod
1-4	-	-	+	Rod
1-5	-	-	+	Rod
1-6	-	-	+	Rod
1-7	-	-	+	Rod
1-8	-	-	+	Rod
1-9	-	-	+	Rod
2-1	+	+	**n	Coccid
2-2	+	+	n	Coccid
2-3	+	+	n	Coccid
2-4	+	+	n	Coccid
2-5	+	+	n	Coccid
2-6	+	+	n	Rod
2-7	+	+	n	Coccid
2-8	+	+	n	Coccid
2-9	+	+	n	Coccid

*: Batch number of isolation and number of isolate in each batch; **: Null

Table 2: Identification details of the isolates

Isolates	Level of ID	Name	Probability (%)	Similarity	Distance between two species
1-1	Species	*Flavimonas oryzihabitans*	100	0.707	7.64-4.45 = 3.19
1-2	Species	*Enerobacter cloacae*	99	0.578	8.20-6.50 = 1.70
1-3	Species	*Sphingomonas paucimobilis* B.	100	0.566	11.25-6.80 = 4.45
1-4	Species	*Sphingomonas paucimobilis* B.	100	0.504	10.90-7.92 = 2.98
1-5	Species	*Sphingomonas paucimobilis* B.	100	0.551	10.14-7.07 = 3.07
1-6	Species	*Sphingomonas paucimobilis* B.	100	0.592	10.33-6.36 = 3.97
1-7	Species	*Sphingomonas paucimobilis* B.	100	0.549	10.17-7.11 = 3.06
1-8	Genus	*Sphingomonas paucimobilis* B.	---	0.317	8.08-7.90 = 0.18
1-9	Species	*Sphingomonas paucimobilis* B.	100	0.551	11.14-7.07 = 4.07
2-1	Species	*Peptostreptococcus hydrogenalis*	100	0.720	7.08-4.22 = 2.86
2-2	Species	*Weissella confusa*	100	0.580	10.51-6.57 = 3.94
2-3	Species	*Weissella paramesenteroides*	100	0.550	10.75-7.14 = 3.61
2-4	Species	*Leuconostoc mesenteroides* ssp. *dextranicum*	97	0.640	6.50-5.21 = 1.29
2-5	Species	*Leuconostoc mesenteroides* ssp. *dextranicum*	100	0.570	9.27-6.71 = 2.56
2-6	Species	*Clostridium innocuum*	100	0.600	8.46-6.22 = 2.24
2-7	Species	*Lactococcus lactis* ssp. *hordniae*	97	0.670	6.24-4.76 = 1.48
2-8	Species	*Weissella paramesenteroides*	97	0.520	8.69-7.34 = 1.35
2-9	Species	*Peptostreptococcus hydrogenalis*	100	0.600	8.95-6.30 = 2.65

Furthermore, all the identified bacteria from the isolation method of lactic acid bacteria were gram positive. With regards to the morphology, all isolates from the first batch and second batch were in rod shaped and coccid, respectively, with the exception of isolate number 2-6 which was rod.

Standard plate count of bacteria for the fresh grass represented an amount of bacteria equal to 2.65×10^5 CFU g^{-1}. Standard plate count of lactic acid bacteria for the fresh grass by considering 3 h activation time in MRS broth and at the temperature 30°C before serial dilution showed that the amount of these bacteria is equal to 8.3×10^3 CFU g^{-1}.

The 18 purified isolates were identified as 9 bacterial species, belonging to *Flavimonas oryzihabitans*, *Enterobacter cloacae*, *Sphingomonas paucimobilis* B. *Weissella confusa*, *Weissella paramesenteroides*, *Leuconostoc mesenteroides* ssp. *dextranicum* and *Lactococcus lactis* ssp. *hordniae*. The *Peptostreptococcus hydrogenalis* and *Clostridium innocuum*, have been isolated when general condition of growth was provided. Identification details of all isolates presented in Table 2.

DISCUSSION

The probability and similarity index must be considered in identification of bacteria by BIOLOG identification system. According to manufacturer's user guide of microlog system (1999), result of the identification is reliable when the similarity is 0.5 and above. Distance is a character which shows how far are between the species of identified bacteria and nearest species and it must be more than 2. According to Table 2, result of all the isolates are trustable except of number 2 and 8 from first batch and number 4, 7 and 8 from second batch. Although the distance in isolate number 1-2 and 2-7 is just under 2, but the result has been accepted because of very good probability and similarity. The bacterial species given as isolate number 1-8, 2-4 and 2-8 are exactly similar to 1-3, 2-5 and 2-3 respectively and hence, their result were accepted. Several reasons such as contamination can affect the bacterial culture to obtain a wrong or inaccurate result.

Flavimonas oryzihabitans was isolated from different brands of mineral water in Australia (Jayasekara *et al*., 1998). It also has been isolated from water, soil and damp environments such as rice paddies and sink drains (Dussart *et al*., 2003). *Flavimonas oryzihabitans* is a yellow pigmented, gram-negative, Oxidize-negative, non fermenting bacillus. This bacterium is an uncommon pathogen which can cause opportunistic infections (Dussart *et al*., 2003).

Enterobacter cloacae previously has been isolated and identified from soil as the cadmium resistant Bacteria (Hu *et al*., 2007). Cadmium (Cd) is a popular heavy metal with the most toxic pollutants of the surface soil layer (Tang *et al*., 2006). Cadmium can be found in the roots of plants which affect the nutrient uptake and homeostasis (Di Toppi and Gabrielli, 1999) and then, animals and humans consume Cd in their diet that can cause diseases (McGrath, 1994).

Using Microorganisms are effective and economical for Bio-sorption properties of cadmium (Esposito *et al*., 2001). Growth of *Sphingomonas paucimobilis* is when cadmium concentration is more than 200 mg L^{-1} and will be inhibited with less than this amount. Living cells of *Sphingomonas paucimobilis* are more able to remove cadmium in compare to nonliving cells. The bacterial biomass can affect the initial pH of solution by the mechanism of cadmium Bio-sorption (Tangaromsuk *et al*., 2002).

Peptostreptococcus hydrogenalis sp. previously isolated from human feces and vaginal discharge which is anaerobic cocci (Ezaki *et al*., 1990). *Clostridium innocuum* which was isolated as the normal human intestinal microflora identifies as Glucoseureide (GU) splitting bacteria (Mohr *et al*., 1999). These two bacteria were died after sub culturing and hence considered as contamination which had been grown by providing semi condition of growth.

Weissella strains have been isolated from a variety of sources. *Weissella paramesenteroides* (formerly *Leuconostoc paramesenteroides*), plays an important role in the first phase of silage fermentation (Dellaglio and Torriani, 1986). *Weisella confusa* has been traced in sugar cane, carrot juice and occasionally in raw milk and sewage (Hammes and Vogel, 1995).

Leuconostoc species are commercially used for the production of glucans (Korakli and Vogel, 2006). Isolation of *Leuconostoc mesenteroides* subsp. *dextranicum* is very popular in starter cultures used in dairy industry (Cogan and Jordan, 1994).

Lactococcus lactis species are common in the production of dairy products. *Lactococcus Lactis* comprises the three subspecies *Lactococcus lactis* ssp. lactis, *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *Hordniae* (Bolotin *et al*., 2001).

Lactic acid bacteria can be divided into two categories based on their pathway in carbohydrate metabolism. The homo-fermentative category comprises of *Lactocococcus*, *Pediococcus*, *Enterococcus*, *Streptococcus* and some *lactobacilli*. The hetero-fermentative category includes of *Leuconostoc*, *Weissella* and some *lactobacilli* (Ross *et al*., 2002).

CONCLUSION

It would be suggested to feed this grass to ruminants in form of silage since the growth of pathogenic bacteria such as *Flavimonas oryzihabitans* or *Enterobacter cloacae* will be inhibited in pH range of 4. All together four lactic acid bacteria were isolated from fresh guinea grass which three of them including *Weissella paramesenteroides*, *Weisella confusa* and *Leuconostoc mesenteroides* subsp. *dextranicum* are hetero-fermentative and one of them, *Lactococcus lactis* ssp. *Hordniae*, is homo-fermentative. Bacterial Inoculants suggested to be used in ensilage of guinea grass since the numbers of naturally occurring lactic acid bacteria are very low.

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