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Full Length Research Paper

Preliminary biological screening of microbes isolated from cow dung in Kampar

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Five distinct morphologically and physiologically isolates were isolated from cow dung at Kampar, Perak, Malaysia and cultured on nutrient agar (NA) plates. Morphological studies including microscopic examination, gram-staining and endospore staining were performed. Isolates K1 and K5 were gram-negative, while isolates K2, K3 and K4 were gram-positive. Isolates K2 and K4 were able to form endospore. Susceptibilities of five isolates to 17 different types of antibiotics were evaluated using the Kirby-Bauer assay. Each individual isolate was resistant to at least 35% of the antibiotics tested. Antibacterial activities against 11 types of test organisms were evaluated. Isolate K4 produced antibacterial agent which inhibited the growth of *Escherichia coli*. Enzymatic assay for the presence of 22 types of enzymes were screened. All isolates produced protease, lipase and esterase lipase. Further studies will be done for identification of all the species.

Key words: Nutrient agar (NA), Kirby-Bauer assay (disk-diffusion assay), antibacterial activities, enzymatic assay.

INTRODUCTION

Cow dung is excreted by bovine animal species which are herbivores. It consists of undigested residues of consumed matter which has passed through the cow's gastrointestinal system. Cow dung is widely studied for its use as organic agricultural fertilizers and extensively explored for its potential as alternative fuel or biogas due to its high methane content (Abdulkareem, 2005). However, there is lack of research on the microbial diversity and other potential applications of cow dung (Yokoyama et al., 2007).

The search for novel antimicrobial agent is one of the current major concerns in medical research due to increasing cases of antibiotics resistance (Chitnis et al., 2000). Cow dung may serve as a source for this research as antifungal agents had been successfully extracted from cow dung (Muhammad and Amusa, 2003). Research revealed that *Aspergillus niger*, *Trichoderma harzianum*, *Bacillus cereus* and *Bacillus subtilis* isolated from cow dung can reduce the growth of *Sclerotium rolfsii*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Helminthosporium maydis* and *Rhizoctonia solani* with inhibitory zones of up to 58%. Furthermore, *B. subtilis* isolated from cow dung can enhance plant growth, sulphur oxidation, phosphorus solubilisation and was found to produce industrial enzymes such as amylase and cellulase (Swain and Ray, 2006).

David and Odeyemi (2007) had reported a number of antibiotic resistant strains that were isolated from the cow dung at Ado-Ekiti, Nigeria. Various biochemical tests such as gram staining, spore staining, catalase test, motility, acid fast staining, starch hydrolysis and some chemical analysis like nitrogen, phosphorus, carbon contents and so on were carried out on the microbes present in the compost using cow dung as booster in the decomposition of organic material (Adegunloye et al., 2007).

The present study aims to isolate microorganisms from cow dung sample for antibiotic susceptibility, antimicrobial and biochemical studies as well as to identify the

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Abbreviations: NA, Nutrient agar; **P10,** penicillin; **AMP10,** ampicillin; **VA30,** vancomycin; **B10,** bacitracin; **E15,** erythromycin; **C30,** chloramphenicol; **F300,** nitrofurantoin; **AK30,** amikacin; **K30,** kanamycin; **N10,** neomycin; **S10,** streptomycin; **CN10,** gentamycin; **TE30,** tetracycline; **NA30,** nalidixic acid; **NOR100,** norfloxacin, **RL100,** sulphanethoxazole; **W5,** trimethoprim.

Table 1. Morphological characteristic of five isolates from cow dung.

Characteristic	Isolates				
	K1	K2	K3	K4	K5
Form of colony	Circular	Circular	Circular	Circular	Circular
Translucency and opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation of colony	Convex	Flat	Convex	Flat	Flat
Margin of colony	Curled	Undulate	Entire	Entire	Entire
Surface of colony	Smooth	Smooth	Smooth	Concentric	Smooth
Pigmentation	Light orange	White	Light yellow	Orange	White
Cell shape	Coccus	Bacillus	Coccus	Bacillus	Coccus
Gram stain reaction	-	+	+	+	-
Spore stain	No	Yes	No	Yes	No

potential applications of cow dung in food, medical, industrial and other fields.

MATERIALS AND METHODS

Source of sample

Cow dung sample was aseptically collected from Jalan Kampar Perdana, Taman Kampar Perdana, 31 900. It was preserved in phosphate buffer solution in sterile Schott bottle.

Isolation

The cow dung sample was serially diluted with phosphate buffer solution to obtain $1:10^6$. The diluted sample was spread evenly on nutrient agar (NA) surface accordingly to the spread plate method (Talaro, 2009). The plates were incubated at 30℃ overnight. After this, five different isolates were selected and streaked on prepared nutrient agar plates based on streak plate method (Talaro, 2009).

Morphological and biochemical examination of the respective isolates

The morphological examinations of the isolates were determined by the standard procedure of basic stain, gram-stain and endospore stain (Talaro, 2009). All isolates were screened for the presence of protease, α-amylase and phospholipase using methods as described by Talaro (2009). The API-ZYM assay was also carried out according to the procedure described by the supplier, BioMeriux. The API stripes were incubated for 4 h and results were determined by the level of colour intensity.

Determination of antibiotic susceptibility (Kirby-Bauer method)

Pure culture colonies of the isolates were inoculated in nutrient broth at 30℃. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the NA plates. The inoculated plates were allowed to dry before placing the diffusion discs containing antibiotics.

Susceptibility of the isolates to 17 types of antibiotics was performed by the disc method as described by James and Natalie (2008). Using commercially available antibiotics discs (Oxoid, UK) containing penicillin (P10), ampicillin (AMP10), vancomycin (VA30), bacitracin (B10), erythromycin (E15), chloramphenicol (C30), nitrofurantoin (F300), amikacin (AK30), kanamycin (K30), neomycin (N10), streptomycin (S10), gentamycin (CN10), tetracycline (TE30), nalidixic acid (NA30), norfloxacin (NOR100), sulphanethoxazole (RL100) and trimethoprim (W5), they were placed on the surface of the agar plates and incubated at 30℃ for 24 h.

Inhibition zone diameters were measured with the diameter of the discs accordingly to the results expressed as susceptible/sensitive (≥21 mm), intermediate (16 to 20 mm) and resistant (≤15 mm) by Liasi et al. (2009).

Assay for antimicrobial agent

All isolates were screened for antibacterial activity against 11 test organisms using the agar well diffusion as described by John *et al*. (2009). Test organisms included seven Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and *Bacillus sphaericus*) and four gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella*). The plates were incubated at 30°C for 24 hours. The inhibitory spectrum of the antibacterial agents against Gram-positive and Gram-negative bacteria were determined.

RESULTS AND DISCUSSION

From the morphological examination, two isolates (K1 and 5) were gram-negative and coccus. Three isolates (K2, K3 and K4) were Gram-positive. Isolates K2 and K4 were bacillus and able to form endospore (Table 1 and Figure 1).

All isolates were evaluated for susceptibility to 17 types of different antibiotics using Kirby-Bauer method. Table 2 showed that all isolates were not susceptible to P10, AMP10, B10, C30, F300 and N10. All isolates were susceptible to NOR10 and RL100. Figure 2 demonstrated that isolate K2 (53%) has the highest susceptibility to all antibiotics tested, followed by isolate K4 (47%), isolate K3 and isolate K5 (35% each), and isolate K1 (29%). All isolates were resistant to 45% of antibiotics tested. *E. coli* and *B. subtilis* were used as control in this study. All isolates were elucidated for antibacterial activities against 11 test organisms. Table 3 showed that only isolate K4 was

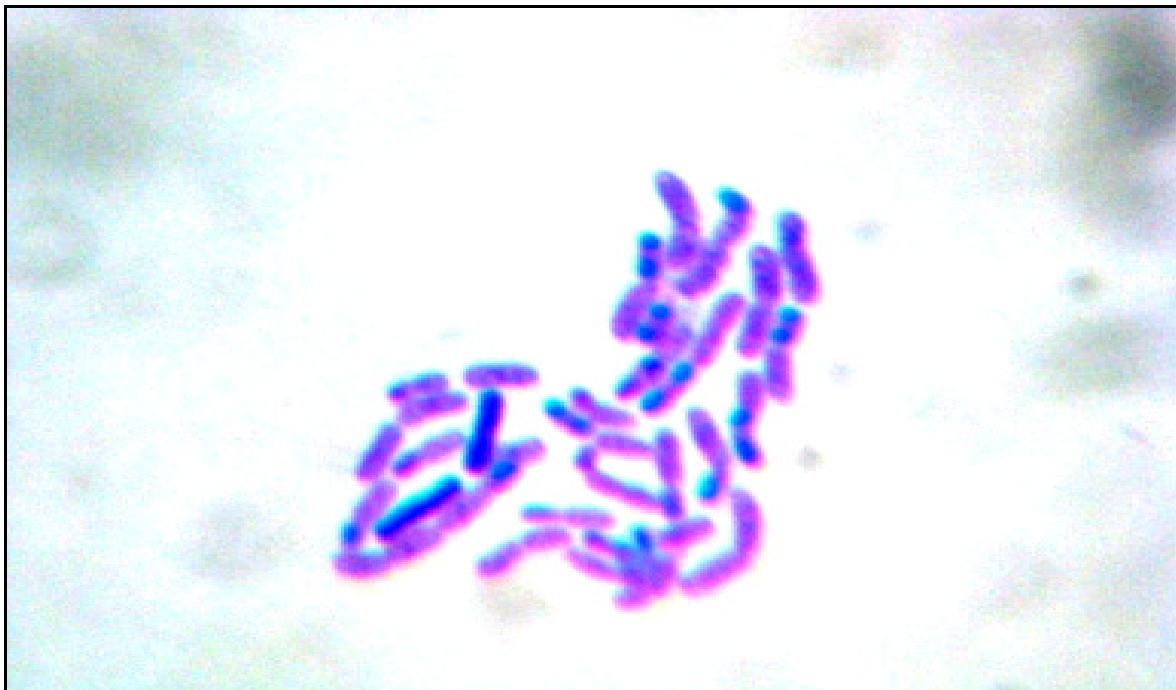

Figure 1. K4 stained with endospore stain (total magnification: 1000X).

Table 2. Assessment of five isolates and two controls on 17 different types of antibiotics.

Isolate / Antibiotic	K1	K2	K3	K4	K5	Control 1 (*E. coli*)	Control 2 (*B. subtilis*)
P10	++	+++	+++	+++	+++	+++	+++
AMP10	++	+++	+++	+++	+++	+++	+++
VA30	+++	+	+	+	++	+++	+
B10	+++	+++	++	+++	+++	+++	+++
E15	+++	+	+++	++	++	+++	+++
C30	+++	++	+++	+++	+++	+	+
F300	+++	+++	+++	+++	+++	+	++
AK30	+++	+	++	++	+	+	+
K30	+	+++	+++	++	+++	+	+
N10	+++	+++	++	+++	+++	+	+++
S10	+++	++	+	+	++	+	+++
CN10	+++	+	+	+	+	+	+
TE30	+++	+	+	+	+	+	+
NA30	+	+	+++	+	+	+	+
NOR10	+	+	+	+	+	+	+
RL100	+	+	+	+	+	+	+
W5	+	+	+++	+	+++	+	+

Degree of susceptibility: + = susceptible (≥21 mm); ++ = intermediate (16 to 20 mm); +++ = resistant (≤15 mm). **P10,** penicillin; **AMP10,** ampicillin; **VA30,** vancomycin; **B10,** bacitracin; **E15,** erythromycin; **C30,** chloramphenicol; **F300,** nitrofurantion; **AK30,** amikacin; **K30,** kanamycin; **N10,** neomycin; **S10,** streptomycin; **CN10,** gentamycin; **TE30,** tetracycline; **NA30,** nalidixic acid; **NOR100,** norfloxacin, **RL100,** sulphanethoxazole; **W5,** trimethoprim.

able to inhibit the growth of *E. coli*. Table 4 showed that all isolates were positive for the presence of protease. Negative results were obtained for the presence of phospholipase and α-amylase. Enzymatic assay was

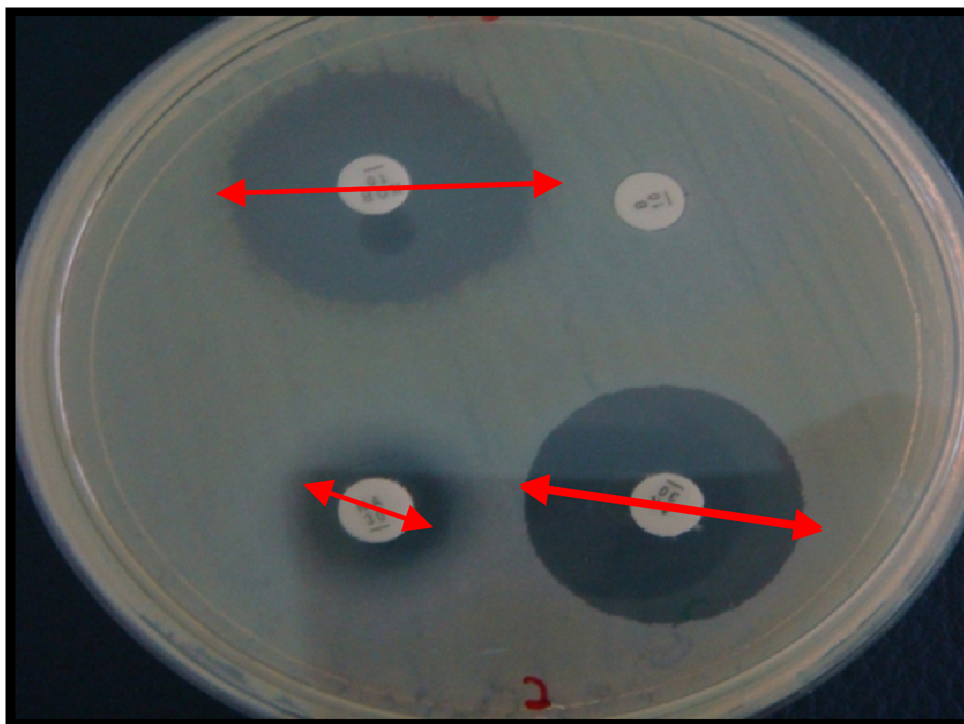

Figure 2. The measurement of inhibition zone on Mueller-Hinton agar.

Table 3. Antibacterial assay of five isolates on 11 test organisms.

Isolate / Bacteria	K1	K2	K3	K4	K5
Gram-positive					
S. aureus	-	-	-	-	-
B. subtilis	-	-	-	-	-
B. cereus	-	-	-	-	-
S. epidermidis	-	-	-	-	-
M. luteus	-	-	-	-	-
E. faecalis	-	-	-	-	-
B. sphericus	-	-	-	-	-
Gram-negative					
P. aeruginosa	-	-	-	-	-
E. coli	-	-	-	++	-
Salmonella	-	-	-	-	-
P. vulgaris	-	-	-	-	-

Degree of inhibition: + = moderate inhibition zone (6 to 9 mm); ++ = strong inhibition zone (10 to 14 mm); +++ = very strong inhibition zone (15 to 18 mm); - = no inhibition zone.

further carried out using API-ZYM kit to screen for the presence of 19 different types of enzymes. Table 5 showed that all isolates were negative for the presence of α-galactosidase, β-glucuronidase, β-gluco-sidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. All isolates were positive for the presence of esterase and esterase lipase. In conclusion, isolate K1 produced the highest activities among the 22 types of enzymes screened which constitutes 50%, followed by isolate K2 (47%), isolates K3, K4 and K5 (40% each).

Isolates K2 and K4, both Gram-positive micro-organisms were capable of forming endospore (Cavaletti et al., 2006). They were also found to produce protease which might be involved in sporulation process.In the present study, all isolates were not susceptible at all to the β-lactam group (P10 and AMP10), polypeptides (B10), fenicols (C30), nitrofurantoin (F300) and one of the aminoglycosides antibiotics (N10). The β-lactam and polypeptide group inhibits cell wall synthesis and have a lethal effect on gram-positive bacteria as they are bactericidal (Liasi et al., 2009). The fenicols inhibit peptidyl transferase of bacterial, ribosome prevents pep-tide bond formation, neomycin (N10) interferes with protein synthesis by binding to 30S ribosomal subunit and nitrofurantoin acts by damaging bacterial DNA (Liasi et al., 2009). All isolates were resistant to almost half (45%) of the antibiotics tested. Each individual isolate had shown at least 35% of resistance to the 17 types of antibiotics tested. This could be a concern for the emergence of antibiotics resistant microorganism. Anti-biotic resistance may occur due to natural processes such as transformation, transduction and conjugation, or due to human mediated activity such as antibiotics abuse, particularly in farming and agricultural industry (MacLean

Table 4. Enzymatic assay of five isolates and two controls.

Enzyme / Isolate	Protease	Phospholipase	α-Amylase
K1	++	-	-
K2	++	-	-
K3	++	-	-
K4	+	-	-
K5	+	-	-
Control 1 *(E. coli)*	-	-	-
Control 2 *(B. subtilis)*	-	-	-

Degree of inhibition: + = moderate clear zone (6 to 9 mm); ++ = strong clear zone (10 to 14 mm); +++ = very strong clear zone (15 to 18 mm); - = no inhibition zone.

Table 5: API-ZYM assay of five isolates

Isolate / Enzyme	K1	K2	K3	K4	K5
Control	0	0	0	0	0
Alkaline phosphatase	1	2	2	0	0
Esterase	4	4	3	4	3
Esterase Lipase	5	3	2	3	3
Lipase	2	1	0	1	0
Leucine arylamidase	5	5	0	4	2
Valine arylamidase	3	1	0	1	1
Cystine arylamidase	1	1	0	1	0
Trypsin	0	0	1	0	2
α-chymotrypsin	1	0	0	5	1
Acid phosphatase	1	2	4	0	1
Naphthol-AS-BI-phosphohydrolase	1	2	1	0	1
α -galactosidase	0	0	0	0	0
ß-galactosidase	0	0	2	1	0
ß-glucuronidase	0	0	0	0	0
α -glucosidase	0	0	3	0	0
ß-glucosidase	0	0	0	0	0
N-acetyl-ß-glucosaminidase	0	0	0	0	0
α -mannosidase	0	0	0	0	0
α -fucosidase	0	0	0	0	0

Number indicates intensity of colour which is proportional to concentration of enzyme.

et al., 2010).

E. coli is one of the major medically important organisms that causes urinary tract infection as well as food poisoning and diarrhea (Alteri et al., 2009). Antibacterial assay revealed that isolate K4 was able to produce antimicrobial agent which inhibited the growth of *E. coli*. Thus, isolate K4 may have potential applications in pharmaceutical industry. The antimicrobial agent produced can be further analyzed for its possibility to be used as therapeutic agent.

All isolates were able to produce protease, esterase and esterase lipase. Protease is an enzyme that catalyses proteolysis which breaks down proteins to its respective amino acids. In addition, they have a variety of applications mainly in the detergent and food industries. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Gupta et al., 2002). Esterase and esterase lipase belongs to hydrolase enzyme which splits esters into acids and alcohols in a chemical reaction of hydrolysis process involving addition of water molecules (Marler et al., 1984). Thus, all isolates may have potential applications in the detergent industry as they are able to remove protein and oil based stains. Isolate K1 yielded the highest number of positive results (50%) for all the 22 types of enzymes screened. However, enzymes produced by isolate K1 might not be suitable for extraction due to the infection caused by the isolate during handling and processing or passed to consumer which might be difficult to treat as the isolate has a low susceptibility to antibiotics tested which is only 29%. On the other hand, isolate K2 was positive for many types of enzymes which account to about 47%. It had shown high susceptibility to antibiotics which account to about 53%. For these reasons, it is more suitable and has potential for enzyme extraction for industrial applications as it is less risky when compared to isolate K1. Moreover, isolate K2 was able to form endospore, it can retain its enzyme synthesis ability despite the harsh conditions in industrial processing.

Further studies can be done to identify the species of all the isolates. The most widely used method for identification is genetic techniques such as the 16S rRNA gene analysis for phylogenetic study (Rasheed et al., 2009). Antimicrobial agent and enzymes produced by the isolates can be screened for possible applications and mass production. They must be evaluated for their safety and toxicity to human, animals and the environment.

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