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ISOLATION AND IDENTIFICATION OF CELLULASE PRODUCING BACTERIA ISOLATED FROM THE RUMEN FLUID OF IRAQI CAMELS

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Abstract

Lingo-cellulolytic bacterial strains have been isolated from rumen fluid of Iraqi Camels and inoculated into BB medium, clear colonies were transferred to Nutrient agar. Biochemical test was conducted for all isolated bacterial such as catalase, oxidase, motility, starch hydrolysis, and sugar fermentation were adopt in this study. Isolated bacteria were inoculated in an enriched media containing MRS agar and Carboxymethyl cellulose (CMC). The result revealed the colonial characterization such as shape, color, size, elevation margin, and gram reaction was determined for each of the isolates. A total of six isolates were identified, five were identified as gram negative while one as gram positive. All isolated bacteria 47B , 26HS, 33b, 35AB, 31A and 2MRS produce cellulase after CM Case activity and isolated bacteria 26HS was showed highest ability to produce cellulase enzyme . Seven different strains isolated on MRS agar identified as lactobacillus strains and were characterized morphology and biochemical characteristics. It has concluded that rumen content of camel considered as a good source of wild strain bacteria capable to produce Cellulase.

Key words: Rumen bacteria, camel, *Bacillus* spp, rumen, *lactobacillus, lingo*-cellulolytic

Introduction

Rough ages play an important role in the cost of ruminant nutrition. However, animal feed costs account for nearly 75% of the total input costs (Moore and Jung, 2001). The rumen is a suitable place for the development of a large number of microorganisms (Pourazad *et al.*, 2015). Mainly consist of three groups of microorganisms bacteria, protozoa and fungi (Theodorou and France, 2005; Krause *et al.*, 2014). Bacteria are the predominant fibrolytic microorganism in the rumen (Sun *et al.*, 2008; Kobayashi, 2006; Chen and Weimer, 2001; Weimer, 1998; Varel and Dehority, 1989). Cellulose is the most abundant polymer found on the world. Different types of bacteria found in the rumen that degrade cell wall components of plants through cellulase enzyme (Suen *et al.*, 2011) convert it into energy source and provide glucose, cellobiose and oligosaccharide hence, cellulase considered as a key in biomass utilization (Shanker and Isaiarasu, 2011). Cellulases are the most important among the industrially hydrolytic enzymes with great significance in present day biotechnology (Haight, 2005; Azzaz, 2009). These enzymes are used to improve feed for animals. Camels have the ability to digest the low-quality shrub, trees and hard plant (Philips *et al.* 2001). Like ruminants, camels have a complex rumen microflora that includes bacteria, archaea, protozoa, and fungi to coordinate plant biomass breakdown (Philips *et al.*, 2001). Over a long period of time, the microbiology of the rumen of animals widely studied and reviewed by many researchers but studies on rumen microbes of camels are very limited. The present study aimed to isolate celluolase producing bacteria from the rumen fluid of Iraqi camels.

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Materials and methods

Samples collection

Camels found in Al-Najef provenance desert were chosen for this study because they were naturally grazing on the rough ages, hard plants and grass in the desert. Fifty five fresh rumen samples were collected from different camels in slaughter house of Al-Najaf during the September, 2016; samples were collected from rumen of camel in the morning after slaughtering in clean

containers size 50ml and all samples were covered with sterilized oil paraffin then immediately transported to the laboratory of Applied Microbiology Department/Food and Biotechnology Center/Directorate of Agriculture Research/Science and Technology.

Isolation of cellulase producing bacteria

Enrichment technique was applied for the first isolation of bacteria from rumen fluid. Bryant and Burkey (BB broth) (castine 1.55%, yeast extract 0.5% ,beef extract 0.75 %, sodium acetate 0.25%, cystein hydrochloride 0.00025%, sodium thiglycollate 0.02%, Agar 0.075% and sodium lactate 0.3%) were dissolved in 100 ml of dissteld water; this broth was used forisolation of anaerobic bacteria. Only 0.1ml from each rumen sample was transferred using sterile pipette into 9.9 ml sterilized BB broth medium, covered with liquid oil paraffin to minimize oxygen then incubated in the anaerobic jarat 37^0C for 18h. The growth of bacteria that developed was subjected to other treatments. One portion of growth was streaked over BB agar and MRS agar (peptone water 1% , beef extract 1%, Yeast extract 0.5 % , dextrose 2%, ammonium citrate 0.2 %, sodium acetate 0.5, magnesium sulphate 0.01%, Manganese sulfate 0.005%, K_2HPO_4 0.2 % and agar 1.5 % all components dissolved in 100ml dissteld water) to isolate different type of bacteria and *Lactobacillus* spp respectively ((Subbarao, 1993, Chandra *et al.,* 2007, Bholay *et al.,* 20 12. Huang *et al.* 2013, Sasikumar *et al.,* 2014) While, second portion of growth was heat treated at 80^0C for 30 min to district vegetative cells and activate spores to isolate spore forming bacteria. Heat treated growth was streaked at top of nutrient agar; all plates were incubated anaerobically at 37^0C for 18h. Well separated isolates were cultured into BB agar, MRS agar and nutrient agar plate until a pure culture was obtained.

Each bacterial isolate was tested for cellulase activity using Carboxymethyl cellulose (CMC) agar plates ($(NH_2)2SO_4$ 0.1, $MgSO_4.7H_2O$ 0.1 %, $CaCl_2$ 0.1%, $FeCl_3$ 0.02, Casein hydrolysate 0.2 %, CMC 1.5 %, K_2HPO_4 0.1%, Agar 1.5 % all components was dissolved in 100ml of distilled water); bacterial growth were streaked using cotton swap on the CMC agar, allowed to incubate for 24- 72 h at 37^0C at anaerobic conditions (Ariffin *et al.*, 2006). Meanwhile, iodine solution was poured into plates and left for 10 minute, positive reaction was indicated by the appearance of cleared zone around bacterial growth in a midst of a brown color; each transparent diameter was measured proportional to the bacterial growth diameter to screen cellulase activity.

Production of Cellulase enzyme

The pure isolates were grown in BB broth for 72h, centrifuged at 5000rpm for 15min; supernatants were collected as a crude enzyme and screened for enzymatic activity. The cellulase activity was determined bymeasuring the amount of reducing sugar released from the substrate carboxymethyl cellulose. The enzymatic reaction mixture contained 0.4ml of 1% carboxymethyl cellulose that prepared in 0.1 Mphosphate buffer (pH 7.2), 0.1ml of crude enzyme; the mixture was incubated for 30minutes at 37°C. Meanwhile, 0.2 ml of enzymatic reaction was mixed with 0.3 ml of 3, 5 dinitrosalicylic acid reagent (DNS reagent), boiling for 10 min in the boiling water bath, red color was developed and reducing sugar was estimated by spectrophotometer at a wavelength 540 n magainst reagent blank (Miller, 1972). Standard curve of Glucose was used to determine the concentration of released reducing sugars. The enzyme activity is expressed as International Unit (IU) in which one unit of enzyme activity was defined as the amount of enzyme that released 1 micromol of glucose per minute.

Identification and characterization of cellulose producing bacteria

Bacterial isolates were identified and characterized after staining using Gram stain. Other tests were performed such as catalase, oxidase, urease, lipase, methyl red, motility, starch hydrolysis and sugar fermentation. The tests were carried according to the methods described by Cheesbrough (2006). The isolates were confirmed using Bergey's Manual of deterministic bacteriologynd edition. The best cellulose producing isolate designed as 26 was further identified using the automation identification system vitec. This system includes kits for gram negative bacteria with 64 biochemical test, the results was obtained after only 18 h as indicated by vitec device.

Statistical Analysis: The statistical analysis system-SAS (2012) program was used. Duncan range tests were used to significant compare between means in this study. Statistical model: $Yijk = \mu + Ai + eijk$.

Results and Discussion

One hundred and fifty out isolates separated and screened for cellulase activity**.** They grouped by gram positive (93) isolates, gram negative (57) isolates. Seven strains of *Lactobacillus* spp. distinguished over MRS agar in which all the strains were characterized depending on the basis of their colonies morphology and biochemical characteristics. Their colonies were small transparent on MRS agar, non- spore forming , Gram-positive, five strains were appeared as rod while two strains were pleomorphic appeared as cocci and rods, they were catalase negative as presented in (table 1) this result agree with Guess as and Kihal (2004) who could isolate Lactobacillus from

Table 1: *Lacto Bacillus* spp characterization (Macroscopically and microscopically)

Morphological characterizationl	11MRS	14MRS	MRS15	6MRS1	20MRS	17MRS	13MRS
Gram stain	G+	G+	G+	G+	G+	G+	G+
Shape	Small cocci	Large diplo rod	Large rod	Medium rod	Larg rod	Small cocci	Small rod
Color	white	White	white	white	White	white	White
Margin	Smooth circular	Smooth circular	Smooth circular	Smooth circular	Rough	Smooth circular	Smooth circular
Size	Small	Medium	large	medium	Large	Small	Small
Spore producing	-	-	-	-	-	-	-
catalase	-	-	-	-	-	-	-

Table 2: Morphological characterization of spore forming bacteria.

NO	Morphological characterizatinl	4	14	12	24	25	27	28	33B
1	Gram stain	G+	G+	G+	G+	G+	G+	G+	G+
2	Shape	Medium diplo rod	Medium diplo rod	Medium diplo rod	Medium rod	Larg rod	Small rod	Medium rod	Small rod
3	Color	Milky	Milky	Milky	White	Milky	Milky	White	Milky
4	Margin	Smooth circular	Smooth circular	Smooth circular	Smooth circular	Rough	Smooth circular	Smooth circular	Smooth circular
5	Size	Large	Small	Small	Small	Large	Small	Small	Small
6	Spore producing	+	+	+	+	+	+	+	+
7	Aerobiosis	FA	FA	FA	FA	FA	FA	FA	FA

Table 3: Characterization of cellulase producing bacteria isolated from the rumen of camel.

Biochemical characterization	Isolate					
	26HS	35AB	47B	31	2MRS	33
Gram stain	G-	G+	G+	G-	G-	G-
Cell shape	bacilli	cocci	rod	strepto-cocci	rod	cocci
Aerobiosis	FA	FA	FA	FA	SA	FA
Catalase	-	+	-	+	-	-
Oxidase	+	-	+	+	+	+
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Starch	+	+	+	+	+	+
Pectin	+	+	+	+	+	+
Blood hemolysis	+	+	+	+	+	+
Skim milk	+	+	+	+	+	+

G-= gram negative, G+= gram positive, += positive results, -= negative results, FA= facultative anaerobe, SA= strict anaerobe

rumen fluid. While, (Muna and Adel, 2014) and (Lamia *et al.*, 2016) could isolate different *Lactobacillus* profile from camel milk.

Our results showed that eight well separated spore forming bacterial isolates could grow at anaerobic condition beside aerobic conditions and they are characterized as *Bacillus* spp, they are list in table 2.

The enrichment isolation technique using anaerobic conditions that adopted at present work was suited for the isolation of floated different bacterial groups that inhabitant the anaerobic environment of camel rumen.

At general the rumen is inhabited by many microorganisms that can colonize and grow and are considered as indigenous normal flora in addition, the ruminal flora includes microorganisms do not established colonization and growth in the rumen. They are dormant, transit and derived from ingested food, water and swallowed air or any habitat of the host. Many factors may contribute to have an effect on the rumen microbial community, such as diet, age and health of animal, season, photoperiod, stress level and feeding regimen.

Our results indicated that six bacterial isolates produced cellulase enzyme at different extent; six out of the isolates showed distinguished cellulose degrading

activity at CMC agar as presented in fig. 1.

Fig. 1: Screening of cellulase activity at CMC agar.

Table 4: Cellulase activity of bacterial isolates from rumen of camel.

Rumen sample	U/ml	Significantly
26HS	1.9±0.20[a]	**
35AB	1.4±0.1[c]	**
47B	1.6±0.06[b]	**
31	1.3±0.07[c]	**
2MRS	0.9±0.12[d]	**
33	1.1±0.09[d]	**

BB broth, 48h, 37°C, anaerobic condition

Four of them were gram negative, one was belong to *Lactobacillus* spp. and the last belong to gram positive group, colony shape, cell arrangement and some biochemical test were presented in table 3. The microorganisms in the rumen fluid and their animals live in a symbiotic relationship at strictly anaerobic conditions in which the gas phase of rumen composed mainly of carbon dioxide and methane that produced by microbial activities. However, small amount of O_2 are contaminants from air that enters via feed and water. Ruminants provide nutrition to microflora at rumen and contribute to the maintenance of physical and chemical conditions for optimal microbial fermentation. Conversely, micro organisms provide energy, proteins and vitamins to animal. Beside, bacterial community are not present as single colonies in the rumen instead they interact with each other that take place into all digestive potentials of the microbes based on the characteristic of each species of bacteria. Previous results indicates that rumen of camels has gram negative bacteria are more than gram positive in the sample research area. Neeru and Manjula (2007). reported that rumen of camel contain from different bacteria population that have ability to digestion and degrade of cellulose by production of fibrolytic enzyme.

Estimation of cellulase activity from the six isolates revealed that different activities of cellulase produced to BB broth at anaerobic condition; the activity ranged from 1.9 to 0.9 IU/ml produced by gram negative 26HS isolate and gram positive 2MRS isolate respectively. The results of screening isolated bacteria for cellulase production was presented in table 4.

The final confirmation for cellulase producing isolate 26 using computerized vitec device indicated that strain 26HS may belong to the species of *pseudomonas loutila*.

Many factors may impacts on the microbial cellulase activity, they includes diet feeding, micro environment surrounding bacteria such as pH and minerals this agree with (Kayouli, *et al.* 1993) who refered to that camels browse on a range of forage plants that are of a little nutritional value with high tannin and lignin content.

Conclusion

Depending on the result of this study it can be conclude that rumen of camels were rich and unique place for a large number of celluolytic bacteria which superior and have potential capability to be used in production of cellulase. On other hand, camel rumen can be a good source of *Lactobacillus* spp bacteria that may play a role in the whole fermentation process in rumen and provide a proper environment in rumen because of their benefit as probiotic. This provides an insight into the poorly researched bacterial ecosystem of the camel and lays the foundation for future studied.

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