Cellulose gene expression power of *Fibrobacter Succinogenes* S85 in Egyptian rams and bucks rumen liquor

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Abstract: Two experiments were conducted in this study. The first experiment was carried out to isolate pure colonies of *Fibrobacter succinogenes* S85 from rumen liquor of the adult Egyptian rams and bucks. It was collected by stomach tube from 3-5 years old rams and bucks at the Agriculture Experimental Station, Faculty of Agriculture, Cairo University, under sterilized conditions. In the second experiment, designations of primers for the cellulase gene of *Fibrobacter succinogenes* S85 strain were performed. Real Time PCR (RT-PCR) and electrophoresis separations were done to assure the target strain in order to quantify the cellulase gene expression in *Fibrobacter succinogenes* S85. According to the conditions of these experiments, it was concluded that, the high digestion efficiency in bucks than rams is due to the cellulose gene expression in *Fibrobacter succinogenes* S85 derived from adult bucks rumen liquor being 3.24 fold higher than the same from adult rams' rumen liquor.

[Salem M.S, **Cellulose gene expression power of *Fibrobacter Succinogenes* S85 in Egyptian rams and bucks rumen liquor]**. *J Am Sci* 2013;9(7):45-53]. (ISSN: 1545-1003). http://www.americanscience.org. 5

Key words: *Fibrobacter succinogenes* S85, Real Time PCR, cellulase gene expression,

1. Introduction

Ruminant animal production contributes substantially to the world's human food supply. This is mainly due to the ability of ruminants to convert carbohydrates found in the fibrous parts of plants to high quality human foods. Ruminant production in dry tropical regions is commonly limited by ability of rumen microorganisms to break down the plant cell wall and ferment the carbohydrates (**Bonnet *et al.*, 2009**).

There are four factors regulating ruminant fiber digestion which are (1) plant structure and composition, which regulate bacterial access to nutrients; (2) nature of population densities of predominant fiber-digesting microorganisms; (3) microbial factors that control adhesion and hydrolysis by complexes of hydrolytic enzymes of the adherent microbial populations, and (4) animal factors that determine the availability of nutrients through mastication, salivation and digesta kinetics (**Gabriella and Kolver 1997and Bonnet *et al.*, 2009**).

The rumen bacterial flora contains over 300 species that vary in their primary role and range of substrate utilized. *Fibrobacter succinogenes* S85 is an important member of the rumen microbial community, a pure culture studies have shown that *F. succinogenes* S85 is a highly cellulolytic mesophilic bacterium capable of growth on crystalline cellulose with a maximum specific growth rate. It is considered the most effective among the rumen bacteria in the utilization of cellulose from plant tissues **(Shinkai *et al.*, 2010).** *Fibrobacter succinogenes* S85 is one of the rumen bacteria that are most active in growth related degradation of recalcitrant forms of cellulose.

The ruminal ecosystem comprises a diverse, symbiotic population of obligately anaerobic bacteria, fungi and protozoa **(Forsberg and Cheng, 2008)** that have adapted for survival in the face of different environments which produced different types of feed. One of the reasons for *Fibrobacter succinogenes* S85 predominance in rumen is its ability to readily degrade various form of crystalline cellulose **(Lynd *et al.*, 2002).**

Grass hay was consumed more by bucks than rams, but digestibility coefficients were similar between species except for crude fiber, which was digested better in bucks **(Gihad, and El-Bedawy 1980).**

The objectives of this study were to isolate one of the common cellulolytic bacteria, *Fibrobacter succinogenes* S85, from rumen of Egyptian rams and bucks and to clarify if their genetic reasons for increasing fiber digestibility in goat comparing with rams or not by using molecular genetics techniques (Real time PCR).

Key words: *Fibrobacter succinogenes* S85, rumen liquor, fiber digestibility, and Real time PCR

2. Material and Methods:

Experimental Design:

The present study comprises two experiments, the 1st to isolate *Fibrobacter succinogenes* S85 from Egyptian rams and bucks rumen liquor, and the 2nd for characterizing its cellulase gene expression.

The first Experiment (Isolation of *Fibrobacter succinogenes S85* from rumen liquor):

2.1. Isolation procedures.

Rumen digesta (500 g) were withdrawn after 4 hrs after feeding through the stomach tube from five

Osemi rams and five Baladi bucks (4-years-old male maintained on a diet of mixed hay *ad libitum* for a month before the rumen liquor sampling, then kept in flasks that were filled to the top and plugged with rubber stoppers to minimize the air contact with the samples. Those samples were used for isolation of cellulolytic anaerobes after enrichment of serum bottle 250 ml capacity filled with, the modified, medium 10 supplemented with cellulose powder as the sole carbohydrate source.

All ingredients were dissolved in distilled water and boiled for 10 min under a stream of oxygen-free CO_2, and cooled under the same phase. The final pH was adjusted at 6.8 – 7.0 and autoclaved at 121°C for 20 min. The ingredients of the medium were prepared according to **Holdman *et al.* (1977),** and it has the following composition in gram or ml per 100 ml:

2.1.A. Media

The medium and the anaerobic dilution solution were prepared under oxygen-free gassing, according to Hungate technique **(Bryant, 1972).**

2.2. Anaerobic dilution blank solution

This solution was used for preparing the dilution series in the isolation trails. It had composition according to **Holdman *et al.* (1977)**

To avoid precipitation, $CaCl_2$ and $MgSO_4$ were mixed first in 300 ml distilled water until it was completely dissolved. Then 500 ml distilled water was added and the remaining salts were slowly added while swirling. Then, the volume was adjusted to one liter (sterilized distilled water). To prepare the dilution blank solution, 50 ml of the salts stock solution, plus 50 ml water containing 0.2 g gelatin and 0.4 ml of resazurin 0.025% solution were mixed together. The solution was usually still slightly pink even after adding the 0.05%, of the reducing agent cysteine. It was added after cooling.

2.2.B. Oxygen removal system

The two required gases for anaerobioses are nitrogen and carbon dioxide. These gases must be purified to be oxygen free, in the sense that oxygen content should be below one ppm, for this purpose, Copper furnace system were used. Copper furnace system is based on the capture of oxygen in the form of copper oxide during the gas flow over the heated (300ºC) copper granules. For this purpose, special apparatus was designed by the other which was a principal investigator for the project of *"Production of Transgenic Aerobic Cellulolytic Microflora to Utilize Farm Waste"* consists of a red copper column, 170 cm in length and about 3 cm in diameter was packed with copper granules. The glass column outer wall was covered with glass wool which was surrounded with a coil of nickel chrome wire as a heater. The coil was insulated by covering it with a long strip of asbestos warped around it. The nickel chrome wire gets its power supply through an auto transformer to raise the inner temperature to 300 - 350ºC which is required to ensure copper oxide formation, leaving the oxygen free gas to flow from the outlet end of the column. The oxygen free gas leaving copper furnace outlet passes through a thick wall black rubber hose to a metallic manifold with six taps. Each tap leads to a hose ending with cotton filled sterilizable syringe ending with a needle for flushing the culture tubes or the medium flask with the oxygen free gas. The final look of oxygen removal system is presented in fig.1.

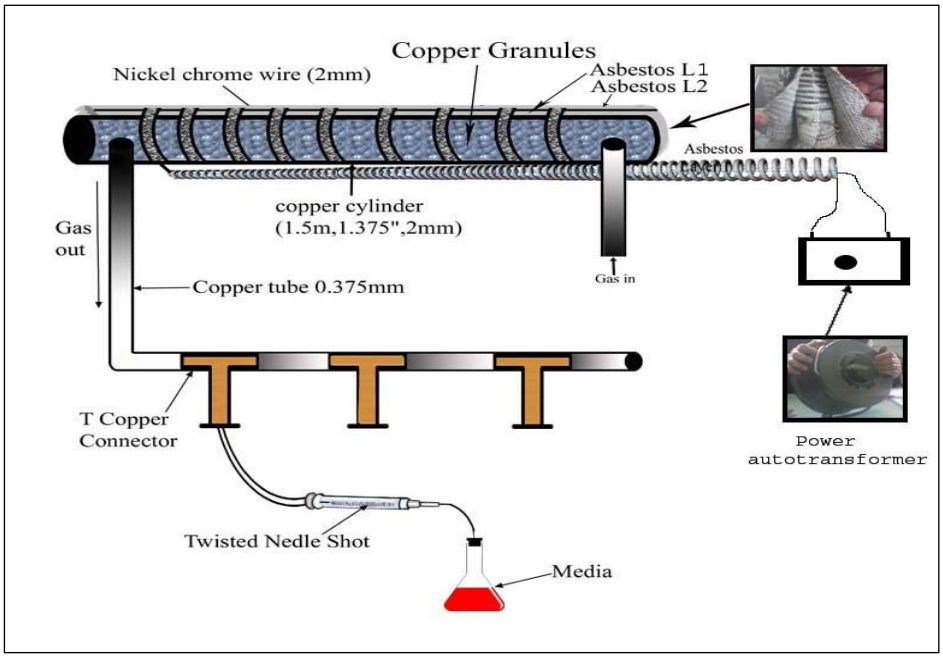


Fig.1. Diagram of oxygen removal system (designed by our lab.)

2.2.C. Isolation of cellulolytic bacteria

All procedures and tools were accomplished under oxygen free gassing and sterilization. The fresh rumen contents were used in the isolation trails. The large particles of the contents were avoided after gravity sedimentation. A series of dilution tubes from 10^1 to 10^5 were prepared using 9 ml anaerobic dilution blank solution per tube. One milliliter of clear rumen fluid was used to inoculate 9 ml of the first dilution and thoroughly mixed. Each dilution was used to inoculate the agar roll tubes, which contained 3 ml of the medium no 10 with 2% agar and 5% cellulose powder as the sole carbon source. The agar tubes were kept in a water bath at 47-49ºC. Five agar tubes were inoculated from each dilution with 1 ml per tube. The inoculated tubes were plugged with butyl rubber stoppers under oxygen free carbon dioxide and crimped with an aluminum cap as recommended by **Hungate (1966).**

2.2.D. Isolation of rumen bacteria using the roll tube method

Isolation of rumen bacteria was performed using the roll tube method of **Hungate (1950)** as illustrated by **Shockey and Dehority (1989).**

Experiment 2: Characterization of cellulase gene from *Fibrobacter succinogenes S85*.

This experiment was carried out in the Molecular Biology (Rumen Biotechnology) Lab **(MBL)** belonging to the project of "Production of Transgenic Aerobic Cellulolytic Microflora to Utilize Farm Waste" Animal Physiology lab, Animal Production Department, Faculty of Agriculture, Cairo University. The isolates of *Fibrobacter succinogenes* were subjected to characterization according to their morphological, cultural, physiological and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology (1984), and Bergey's Manual of Determinative Bacteriology (1994). This includes growth at 39ºC for one week in roll tube agar (medium no. 10 modified) under anaerobic conditions as well as clear zone formation.

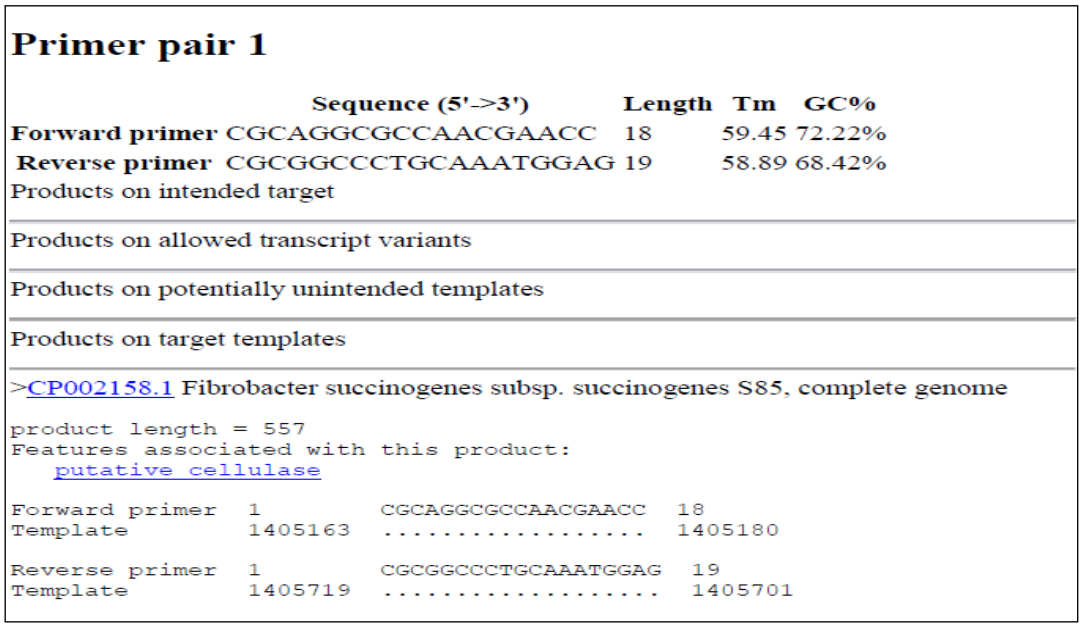
The isolates of *Fibrobacter succinogenes* were subjected to characterization according to their Molecular characteristics in **MBL**, and the part of work about Real Time PCR was done at the Center of Excellence of Biotechnology Research, King Saud University.

2.3.A. Cellulose gene alignment and PCR primer design.

Multiple sequence alignment was performed by CLUSTAL W Multiple Sequence Alignment Program (Higgins *et al.,* 2007) on three species of cellulolytic bacteria, using default parameters. The PCR primer was tested by sequence manipulation suite software (Stothard, 2000), to indicate each primer location on the cellulase gene. Also PCR designed primer was verified by in silico PCR software included in Fast PCR V6.0 package and PCR primer stats in the package of sequence manipulation suite Java script programs. Cellulase gene PCR product was calculated utilizing NCBI primer BLAST server http://www.ncbi.nlm.nih.gov/tools/primer-blast on the genome of *Fibrobacter succinogenes S85*. Furthermore, using this solution for screening any further matches with the designed primers on the most of culturable microorganisms,

(FW Primer was CGCAGGCGCCAACGAACC length 18 Tm 59.45 GC% 72.22%)

(RV Primer was CGCGGCCCTGCAAATGGAG length 19 Tm 58.89 GC% 68.42%)



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CGCAGGCGCCAACGAACC	18	59.45	72.22%
Reverse primer	CGCGGCCCTGCAAATGGAG	19	58.89	68.42%

Products on intended target

Products on allowed transcript variants

Products on potentially unintended templates

Products on target templates

>CP002158.1 Fibrobacter succinogenes subsp. succinogenes S85, complete genome

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product length = 557
Features associated with this product:
   putative cellulase

Forward primer  1         CGCAGGCGCCAACGAACC  18
Template        1405163   ..................  1405180

Reverse primer  1         CGCGGCCCTGCAAATGGAG  19
Template        1405719   ...................  1405701
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Fig. 2. The NCBI primer blast data output of designed cellulase gene

Nucleotide sequence alignment phylogeny analysis was performed by the same program and drew by phylodendron phylogenetic tree drawing software version 0.8d (Gilbert, 1997).

Designed primer calculation resulted as NCBI primer BLAST server output (Fig. 2) at molecular weight of 557 pb, and *Fibrobacter succinogenes* S85 specific product in the putative sequence of cellulase gene (Bioneer corporation, Korea).

2.3.B. Real Time PCR

Synthesized cDNA used as a template to analyze the level of gene expression representative for the quantity of expressed mRNA of the desired gene (Cellulase). Reaction was prepared in volume of 25 µl contained 1X of GoTaq® Colorless Master Mix (Cat# M7132, Promega corporation, USA), 10 pmol primer, and 1 µl from the previous reverse transcription reaction (cDNA) product. Also 1X Syber green reporter dye was included in the reaction for quantify the progression of amplification.

Amplification cycles program is cited up in Applied Biosystems 7500 Instrument guide. First step of pre-denaturation was adjusted at 95ºC for 5 minutes followed by 40 cycles of amplification began at 95ºC for 30 seconds, 56ºC of extension of annealing for 20 seconds and 72ºC of extension for 30 seconds.

The finalization step, adjusted at 72ºC for 10 minutes, end point analysis cycles began with denaturation step at 95ºC for 30 seconds and annealing temperature at 70ºC for 30 seconds. The temperature increased by 0.5ºC per second of ramping for continuous scanning for the decreasing of the reporter dye until it reached 95ºC of complete denaturation. This part of work about Real Time PCR was done at the Center of Excellence of Biotechnology Research, King Saud University, Saudi Arabia.

3. Results and Discussion

Experiment I: Isolation of *Fibrobacter succinogenes* S85 from rumen liquor

Isolation trials were carried out from the rumen contents withdrawn from living mature rams and bucks via stomach tube, four hours after feeding roughages in MBL.

The inoculated tubes were checked for colonies formation after one week of incubation at 39°C and up to one month. Colonies formation, due to the digested cellulose was used as an indicator for the pure strain of cellulolytic anaerobes *Fibrobacter succinogenes* S85 according to **Varel and Yen (1997)** Fig. 3 after colonies formation; these colonies were *Fibrobacter succinogenes* S85**.**

Some tubes (Fig. 4) showed the presence of VFA production between vasper layer and the medium surface indicates a positive growth of *Fibrobacter succinogenes* S85 and been sured that was our target strain by staining with Gram stain and microscopic examination (Fig. 5). The other tubes showed that there was no space between the media and the vasper indicates a negative growth Fig. 4. The negative growth tube by Gram stain and microscopic examination showed a mixture of ruminal bacteria (Fig. 6).

Trials were made for further purification of the isolated anaerobic cellulolytic bacteria. Colonies were picked up anaerobically from agar roll tubes and Petri dishes, transferred into medium no. 10 with 5% cellulose powder as the sole carbon source and incubated at 39°C. Tubes were observed daily, revealing no clear zones were formed after incubation periods up to three weeks.

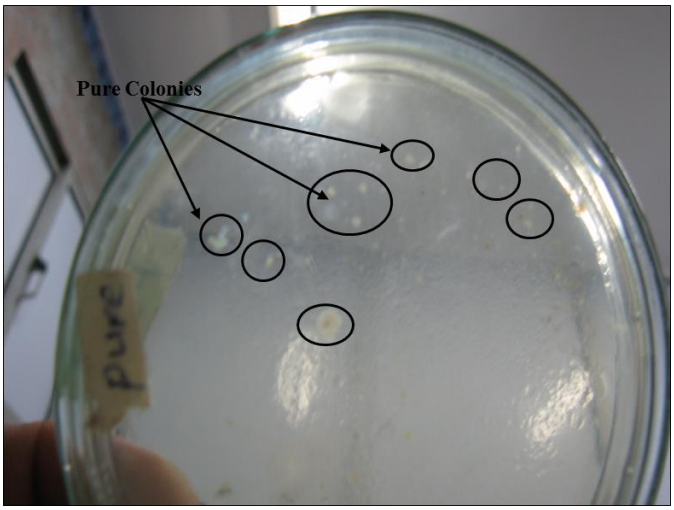
A 

Fig. 3. Colonies of *F. succinogenes* S85 in Petri dishes

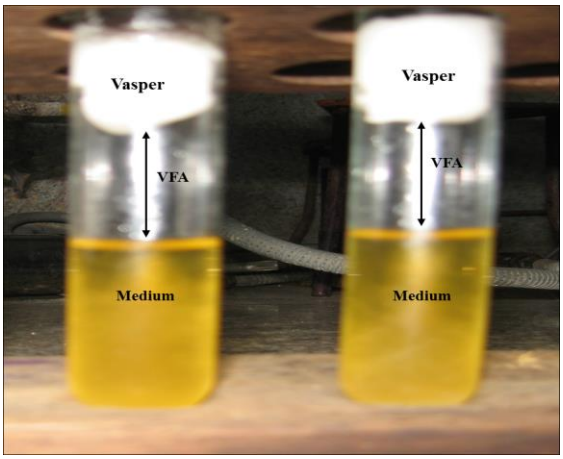
B 

Fig. 4. Growth of *F. succinogenes* S85 and VFA production

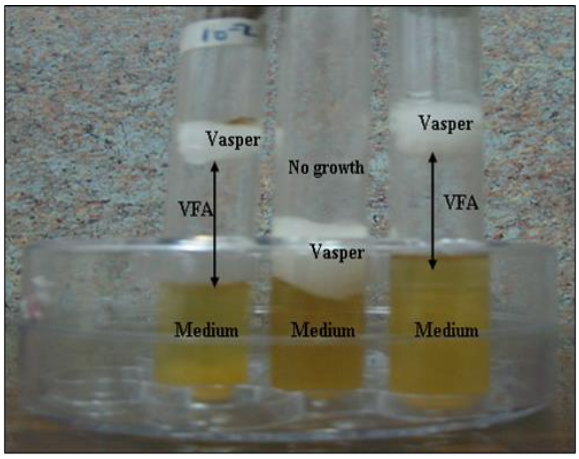
C 

Fig. 5 Production of VFA (positive growth) and non-growth tubes

Plate (Fig. 3) showed colonies formation; these colonies were *Fibrobacter succinogenes* S85, according to Varel and Yen (1997).

Some tubes (Fig. 4) showed the presence of VFA production between vasper layer and the medium surface indicates a positive growth of *Fibrobacter succinogenes* S85. The other tubes showed that there is no space between the media and the vasper indicates a negative growth Fig. (5).

Trials were made for further purification of the isolated anaerobic cellulolytic bacteria. Colonies were picked up anaerbically from agar roll tubes and Petri dishes, then they were transferred into medium no. 10 with 5% cellulose powder as the sole carbon source, and incubated at 39°C. Tubes were observed daily, but no clear zones were formed after incubation periods up to three weeks.

In fact, the cellulolytic bacteria are closely associated with non cellulolytic bacteria as reported by many investigators (Bryant *et al.*, 1971; Smith and Bryant 1979). Pochon (1941) found that in fermenting cellulose, non cellulolytic associated organisms are needed. The types of the mutual interactions between cellulolytic anaerobes and their associates were observed by Latham and Wolin (1979). They found that the growth efficiency of *R. flavefaciens* increased in the presence of *Methanobacterium ruminantium.*

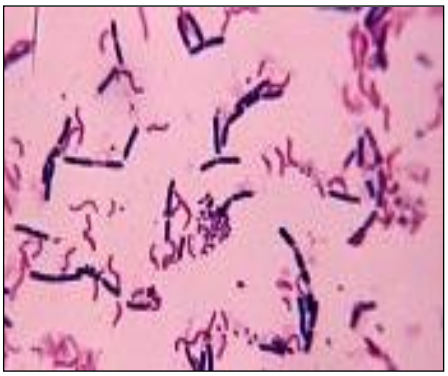


Fig.6. Mixed strains isolated from experimental samples stained with Gram stain

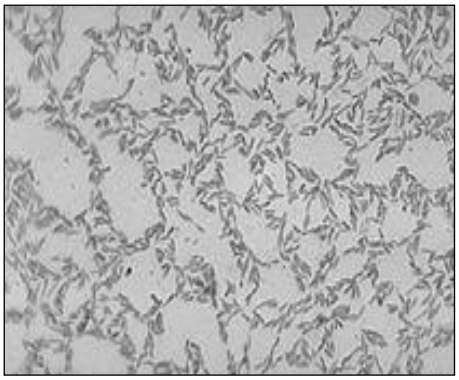


Fig.7. Pure strain of *F. succinogenes* S85 stained by Gram stain

Experiment II: characterization of cellulase gene from *F. succinogenes* S85.

PCR primers. As shown in figure 2, the output results of NCBI primer BLAST server of designed primer, that matched 100% to the cellulase coding sequence of *F. succinogenes*, and predicate 557 bp of PCR product size which is approved practically by the Agarose gel electrophoresis result which shown in figure 7.

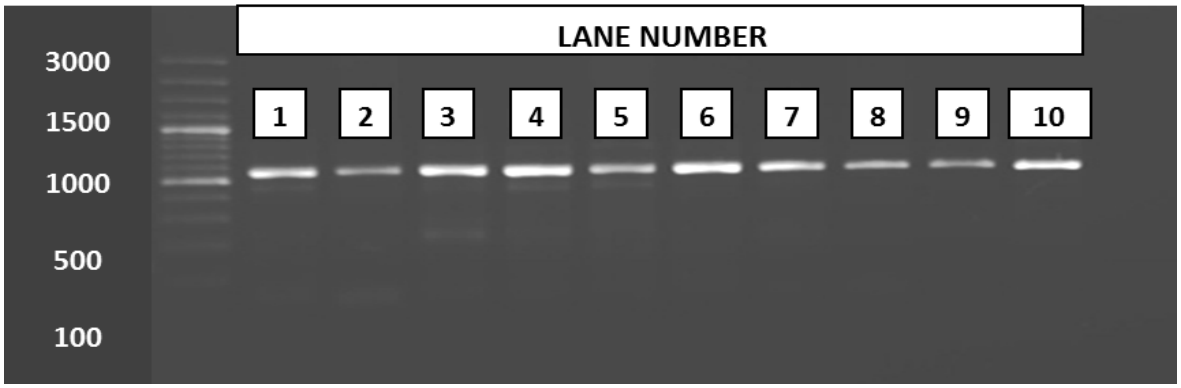


Fig. 8. EtBr stained Agarose gel electrophoresis loaded by cellulase gene PCR products of Goat-derived samples (lane 2,3,4,5,6) and Sheep-derived samples (lane 7,8,9,10), Where the first lane loaded by 100 bp DNA Hyberladder II (Bioline Inc, Gemany). Molecular weight calculation of loaded PCR products indicates to be equal 557 bp.

Real time PCR analysis for *F. succinogenes* S85

Sample	Target	Task	Ct	Nrm. Qty	Tm1	Tm2	Tm3
G1	CELLULASE	UNKNOWN	28.6753		87.6907		
G2	CELLULASE	UNKNOWN	28.3532		87.1649		
G3	CELLULASE	UNKNOWN	29.2978		74.0206	86.5515	83.4845
G4	CELLULASE	UNKNOWN	22.8564		88.3041		
G5	CELLULASE	UNKNOWN	30.367		87.866		
NTC	CELLULASE	UNKNOWN	Undetermined		71.0412		
SH1	CELLULASE	UNKNOWN	28.9667		88.3918	83.5722	
SH2	CELLULASE	UNKNOWN	29.7156		87.866		
SH3	CELLULASE	UNKNOWN	28.1291		87.1649		
SH4	CELLULASE	UNKNOWN	30.7013		87.1649		
SH5	CELLULASE	UNKNOWN	30.5146		87.1649		

Where: G: Goat ruminal samples, Sh: Rams ruminal samples, NTC: Non template control (water) TM: Melting temperature, CT: Cycle threshold

Fig. 9: Captured photo of ABI 7500 instrument output table represents the absolute CT value and melting temperatures of each PCR products of samples

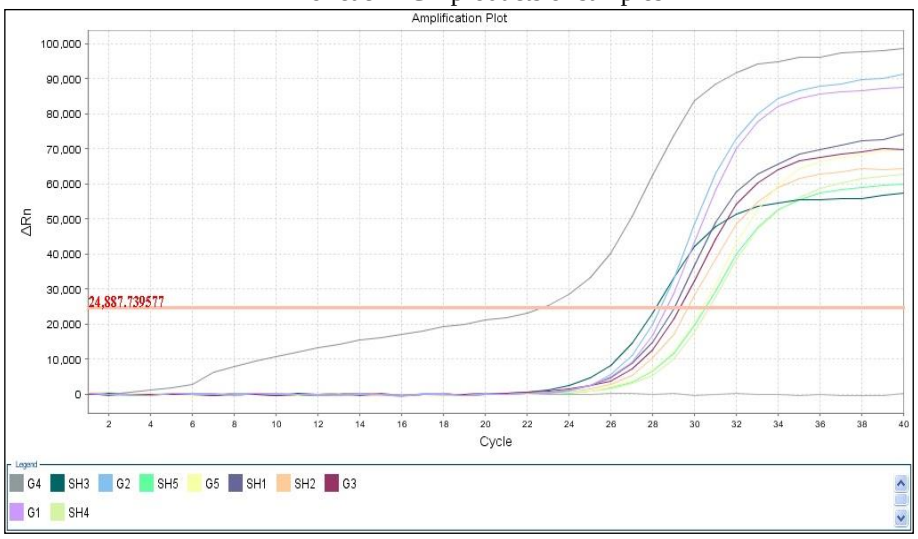


Fig. 10: Captured photo of the amplification plot resulted from cellulase gene expressed in *F. succinogenes* derived from both Goat rumen samples (G samples) and Rams rumen samples (SH samples). The earliest curve is represent Goat sample number 4 which across the threshold line in cycle number 22.8564 indicating the highest expression for the mentioned cellulase gene in this sample.

The Cycle threshold (CT) value-based comparative analysis of a cellulase gene as represented by *Fibrobacter succinogenes* S85 from a cellulase producing bacteria in the rumen is calculated in favor of goat cellulolytic ruminal bacteria. It showed an average CT value in average of 27.91. Rams cellulolytic ruminal bacteria showed an average of CT value 29.61. This indicates that goat rumen bacteria have higher expression of cellulase gene than ram's rumen bacteria.

Amplification plot Indicated highly expressed cellulase gene sample in the Goat 4 sample (CT value = 22.86). On the other hand the melt curve end point analysis shown in Fig. (11). Represents single PCR products except some individuals with noisy background. Both Goat 3 and Rams 1samples did not significantly affecting the single peak shape of the melt curve (Fig. 11). However, four samples shown the same TM value at 87.1649ºC and the others around this TM is proved the reality of presenting single PCR product representing the cellulose gene.

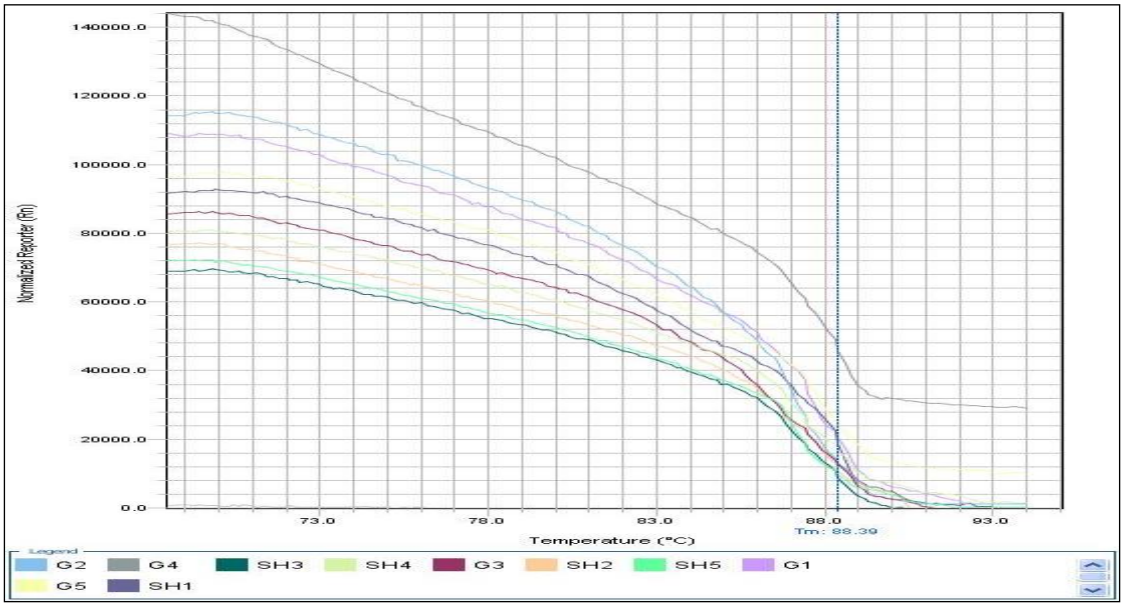
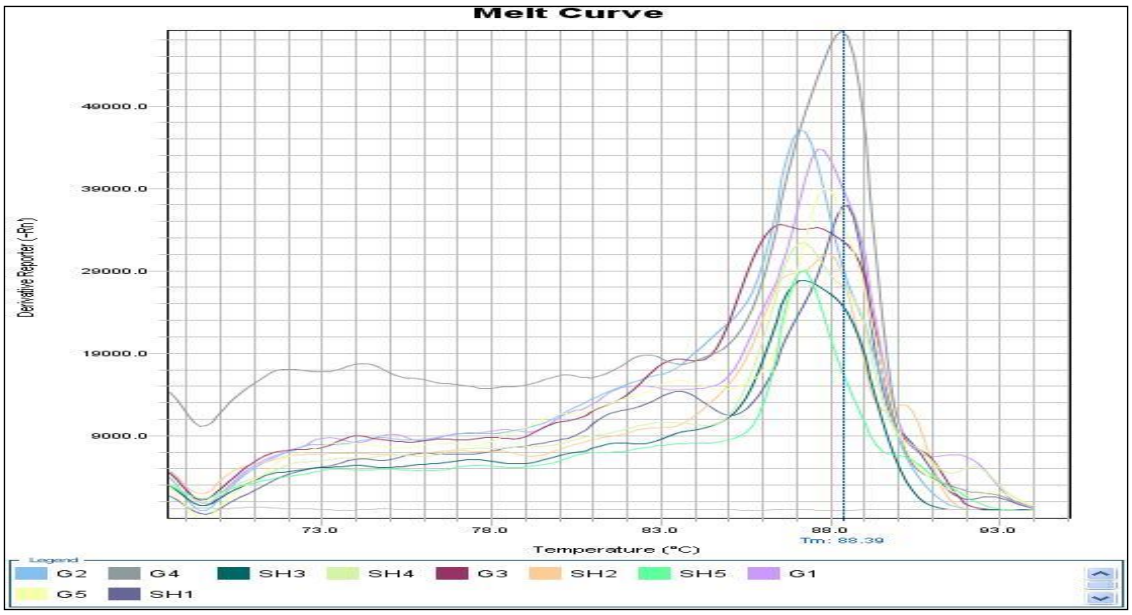


Fig. 11. Melt curve end point analysis (normalized reporter) of *F. succinogenes* S85 in rams and buck samples



G: Goat ruminal samples, Sh: Rams ruminal samples, NTC: Non template control (water), Tm: Melting temperature, CT: Cycle threshold.

Fig.12. Real Time PCR output Table represents CT value and melting temperatures of each PCR products of samples

The CT value-based comparative analysis of cellulase gene represented by *Fibrobacter succinogenes* S85 cells as cellulolytic bacteria in the rumen favored goat rumen bacteria. Results showed that goat rumen derived samples CT value had an average of 27.91. Rams' rumen derived samples shown this average in value of 29.61 (Fig.12). The lower or earlier CT value means a larger amount of starting cDNA copies. These confirm that goat rumen bacteria have higher expression of the cellulase gene than rams' rumen bacteria. A mathematical model was used to compare the level of cellulase gene expression using the equation of **Livak and Schmittgen (2001)** as follows:

$$\textbf{Ratio}_{(\text{Sample1:Sample2})} = \mathbf{2}^{(\Delta \text{ Ct of Sample1} - \Delta \text{ Ct of Sample2})}$$
$$= \mathbf{2}^{29.6054 - 27.9099} = \mathbf{2}^{1.6955} = \mathbf{3.24}$$

Where CT is real time PCR cycle number at which detectable signal of reporter dye is achieved.

The previous equation result indicated that cellulase gene expression in *Fibrobacter succinogenes* S85 derived from goat rumen is **3.24** fold higher than that derived from ram's rumen. Amplification plot Indicated that the highly expressed cellulase gene in the Goat 4 sample (CT value = 22.8564). On the other hand, the melt curve end point analysis shown in (Fig. 11, 12) represent single PCR products except individuals background noises in both G3 and SH1 samples and not significantly affect the single peak shape of the melt curve. However, samples shown the same Tm value at 87.1649ºC and the others around this Tm which proved the reality of presenting of single PCR product represent the cellulase gene.

Up to our knowledge the expression of Celluletic gene was measured and concluded the result in this report. Further experiments needed in order to confirme our results regarding Celluletic genes.

Acknowledgement

Grateful thanks are due to the scientific team of project titled "Production of Transgenic Aerobic Cellulolytic Microflora to Utilize Farm Waste" Supported by grants from Academy of Scientific Research and Technology at Animal Physiology lab, Animal Production Department, Faculty of Agriculture, Cairo University from 2006 to 2011. Also, thanks are extended to Waleed A. El-Nemr, researcher in Center of Excellence of Biotechnology Research, King Saud University, Saudi Arabia for his help in Real Time PCR set up. A lot of appreciation for Dr. Mohamed Nabiel associate professor, microbiology department, National Research Center, Dokki, Giza

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5/23/2013