UNIVERSITI PUTRA MALAYSIA

A STUDY ON ANAEROBIC CELLULOLYTIC BACTERIA ISOLATED FROM RUMINANTS

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by

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The study of rumen microbiology began in the 1940s but the progress made from those years onwards have not been able to match the burgeoning studies in genetic engineering aimed at optimizing the process of cellulose digestion. The basic study of the complex bacterial processes involved in cellulose digestion will provide a rational baseline which is important in such an undertaking. The need to develop simpler and more efficient methods of isolation, characterization, enumeration and maintenance of cellulolytic bacteria are also important because this will provide new strains of organisms that will be very valuable in the determination of the

molecular processes of cellulose digestion and in the development of genetically engineered improvements. In view of this, a project was undertaken to improve the isolation method for cellulolytic bacteria and to study their adaptability and cellulolytic characteristics *in vitro*.

A simple method using medium enriched with Whatman No. 1 filter paper as the sole selective substrate, was very effective in the isolation of cellulolytic bacteria from the rumen of a steer in Canada and a water buffalo in Malaysia. Both of these animals were fed high-fiber rations. The rumen sample from the steer was incubated in the enrichment broth medium for 36 h, then inoculated into non-selective glucose-cellobiose-starch-agar roll tubes. A total of 120 colonies were randomly picked from these roll tubes and 45 of these colonies were found to be cellulolytic. In the same manner, 36 colonies out of 90 colonies obtained from the buffalo were cellulolytic. Most of these isolates were identified as *Bacteroides succinogenes*. *Ruminococcus flavefaciens* was found in lesser numbers, and no *Ruminococcus albus* was isolated. None of the colonies were contaminated with cells resembling *Treponema* sp. but contamination by *Butyrivibrio* sp. was noted. The method described here is effective, requires less time than the conventional cellulose agar method, and is superior to the latter because pure colonies of *B. succinogenes* are detected as being cellulolytic in broth medium while they escape detection because they fail to produce clear zones when grown in cellulose agar roll tubes prepared from Whatman No. 1 filter cellulose paper. *Bacteroides*

succinogenes formed clearings in this type of cellulose agar roll tubes only when contaminated with Butyrivibrio sp. Scanning and transmission electron microscopy of 24-h enrichment cultures of samples from both the buffalo and steer rumen showed that the cellulose filter paper fibers were very heavily colonized by bacteria. They were colonized primarily by a Gram-negative rod and a Gram-variable, slightly elongated coccus and secondarily by a spiral bacterium resembling Treponema bryantii and a slightly curved rod resembling Butyrivibrio sp. A study was also made of the adaptability and characteristics of 37 fresh isolates of cellulolytic rods identified as B. succinogenes and 7 cellulolytic cocci identified as R. flavefaciens isolated from the rumen of a steer. All the Bacteroides cultures showed spontaneous formation of spheroplasts in stationary phase cultures and older cultures in both synthetic and rumen fluid media. When cultures of the 37 strains of B. succinogenes were stored at 4°C, all the strains lost their cellulolytic activity after 10 weeks and viability after 11 weeks of storage. Frequent transfer of cultures of B. succinogenes promoted very high cellulolytic activity, whereas prolonged intervals between culture transfer markedly reduced this capability. Ruminococcus flavefaciens was able to digest cellulose filter paper as well as B. succinogenes but showed a decline in activity after the second day compared to B. succinogenes which showed a decline in activity only after 5 days of incubation when straw substrate was used. None of the B. succinogenes isolates produced clear zones when the cellulose powder was prepared from Whatman No. 1 filter paper although all R. flaveflaciens strains

were able to do so. However, B. succinogenes was able to produce clear zones when the cellulose agar roll tubes were prepared from Avicel microcrystalline cellulose. When B. succinogenes was incubated with filter paper in a liquid medium, the paper was degraded in long, straight lines which eventually fragmented the piece of filter paper, whereas the first signs of cellulolytic attack by R. flavefaciens were small, yellowish, clear, round spots on the paper which subsequently disintegrated to form a viscous lump. These differences in filter paper and cellulose agar roll tube digestion may reflect differences in the cellulase systems of these organisms.

Abstrak daripada tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia untuk memenuhi sebahagian dari keperluan untuk Ijazah Master Sains.

KAJIAN KEATAS BAKTERIA SELULOLITIK ANAEROB YANG DI ASINGKAN DARIPADA RUMINAN

Oleh

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Kajian mengenai rumen mikrobiologi bermula pada tahun empat puluhan tetapi kemajuan yang diperolehi pada tahun-tahun yang berikutnya tidak dapat menandingi dengan kemajuan "genetic engineering" yang bertujuan mendapat pencernaan selulos yang terbaik. Kajian permulaan tentang proses bakteria mencerna selulos yang kompleks akan memberi asas rasional yang penting kepada projek yang tersebut di atas. Keperluan untuk menembangkan kaedah-kaedah yang senang dan lebih efektif untuk mengasing, mengenalpasti,

menghitung dan memelihara bakteria-bakteria selulolitik *in vitro* penting juga kerana ini akan menghasilkan bakteria-bakteria strain yang baru untuk kita menetapkan proses molekular pencernaan selulos dan pengembangan "genetic-engineered" bakteria-bakteria yang lebih baik. Berdasarkan pandangan di atas, satu projek telah dijalankan untuk memperbaiki kaedah pengasingan bakteria-bakteria selulolitik dan untuk mengkaji penyesuaian dan ciri-ciri selulolitik bakteria-bakteria *in vitro*.

Satu cara mudah yang menggunakan medium yang diperkayakan dengan kertas penapis selulos Whatman No. 1 sebagai substrat selektif tunggal didapati efektif untuk mengasingkan bakteria selulolitik daripada rumen lembu di Canada dan kerbau di Malaysia. Kedua-dua haiwan ini diberi makanan serabut. Sampel daripada rumen lembu diramkan selama 36 jam dalam medium cair yang telah diperkayakan, kemudian disuntik ke dalam medium agar glukos-selobios-kanji yang bukan selektif di dalam "roll tube". Sejumlah 120 koloni telah diperolehi secara rambang daripada "roll tube" tersebut dan 45 daripada koloni-koloni itu didapati sebagai selulolitik. Dengan cara yang serupa, 36 daripada 90 koloni yang diperolehi daripada kerbau didapati sebagai selulolitik. Kebanyakan bakteria yang diasingkan itu dikenalpasti sebagai jenis *Bacteroides succinogenes* dan diikuti oleh jenis *Ruminococcus flavefaciens* tetapi jenis *Ruminococcus albus* tidak pernah diperolehi. Koloni-koloni didapati bebas dari pencemaran oleh sel-sel yang menyerupai jenis *Treponema* sp. tetapi pencemaran oleh jenis *Butyrivibrio* sp. dapat dikesan.

Kaedah yang dihuraikan di sini adalah lebih efektif, memerlukan masa yang singkat dan lebih baik jika dibandingkan dengan kaedah agar selulos konvensional. Kaedah ini juga dapat mengesan koloni-koloni jenis B. succinogenes tulin yang menunjukkan ciri-ciri selulolitik ketika di dalam medium cair berbanding dengan kaedah konvensional di mana ciri selulolitik ini tidak dapat di kesan kerana B. succinogenes tidak menghasilkan zon cerah di atas agar selulos "roll tube" yang dibuat daripada selulos penapis Whatman No. 1. Bacteroides succinogenes menghasilkan zon cerah di dalam agar selulos roll tube hanya apabila dicemari oleh Butyrivibrio sp. Pemeriksaan elektron mikroskop daripada sampel rumen yang diramkan selama 24 jam dalam medium cair yang diperkayakan menunjukkan selulos fiber kertas penapis dikolonikan dengan banyak bakteria. Selulos fiber dikolonikan terutama dengan Gram-negative rod atau dengan Gram-variable cocci bersekutu dengan bakteria spiral menyerupai Treponema bryantii dan satu bakteria melentur menyerupai Butyrivibrio sp. Kajian mengenai penyesuaian dan ciri-ciri juga dilakukan ke atas 37 jenis B. succinogenes dan 7 jenis R. flavefaciens berciri selulolitik yang baru diasingkan daripada lembu. Kesemua kultura Bacteroides menunjukan pembentukan spheroplast dengan sendirinya semasa dalam fasa pegun di dalam medium sintetik atau medium cecair rumen. Apabila 37 jenis B. succinogenes itu disimpan pada suhu 4°C, ciri selulolitiknya hapus pada minggu yang ke sepuluh dan viabilitinya hapus pada minggu ke sebelas. Kekerapan pemindahan kultur-kultur bagi B. succinogenes meningkatkan aktiviti selulolitiknya tetapi sekiranya pemindahan

kultur-kultur dilakukan jarang-jarang sekali, ia akan melemahkan aktiviti ini. Ruminococcus flavefaciens boleh mencerna kertas penapis selulos seperti juga B. succinogenes tetapi aktiviti pencernaan itu semakin kurang pada hari yang kedua berbanding dengan B. succinogenes yang kurang pada hari yang kelima diramkan apabila substrat jerami digunakan. Bacteroides succinogenes tidak menghasilkan zon cerah apabila tepung selulos yang digunakan di perbuat daripada kertas penapis Whatman No. 1 tetapi semua jenis R. flavefaciens berupaya menghasilkan zon cerah. Tetapi B. succinogenes boleh menghasilkan zon cerah di dalam agar selulos "roll tube" yang diperbuat dengan selulos Avicel microcrystalline. Apabila B. succinogenes diramkan dengan kertas penapis selulos dalam medium cair, kertas tersebut akan dipecahkan bermula secara garisan yang lurus dan panjang sebelum menjadi hancur. Pemecahan kertas penapis selulos oleh R. flavefaciens pula bermula secara bintek-bintek kecil, bulat dan berwarna kuning di atas pemukaan kertas tersebut yang kemudiannya akan hancur menjadi gumpalan yang pekat. Perbezaan kaedah penghadaman kertas penapis dan agar selulos dalam "roll tube" yang dipertunjukkan itu mungkin mencerminkan perbezaan sistem selulosa di antara organisma- organisma itu.

CHAPTER 1

INTRODUCTION

The study of anaerobes is a very important part of microbiology. Its emergence in the 1940s was due almost entirely to R.E. Hungate whose work on anaerobic rumen microbiology is considered to be a necessary text for all students in this field. Aerobes are organisms which are not subject to irreversible damage by a high redox potential although they contain many systems at low redox potentials. This is because they possess the capability to reduce the potential and maintain it at that level by the continuous interaction with reducing systems in an active cell. Anaerobes are organisms which may be killed by a high redox potential or may not grow unless the medium is in a reduced state. They lack superoxide dismutase reducing enzymes and may be irreversibly destroyed in high redox potential states.

The rumen supports a huge population of mixed microorganisms of which many are obligate anaerobes. They are unable to reduce medium by themselves and exist in an environment where the redox potential is around -400 mV. Therefore in order to culture them either in mixed or isolated strains in vitro, their media has to be reduced for them and to exclude fastidiously the presence of

oxygen. Hungate (1950) was the first to describe anaerobic *in vitro* methods which accomplished a simulation of the natural environment through the exclusion of oxygen, the inclusion of naturally occurring nutrients in the media and the maintenance of a carbon dioxide atmosphere. This reduction process has been modified many times over the years. Our experiments to investigate anaerobic organisms in the rumen were conducted using media developed by Hungate (1947) and modified by Scott and Dehority (1965) termed as Scott and Dehority's artificial modified media, or in short, MOD-SD.

Some anaerobes such as the methane-forming bacteria cannot initiate growth at potentials greater than -330 mV, and by calculation, Hungate (1969) estimated that this would be 10^{-75} of the concentration of oxygen in the atmosphere. Similarly, the amount of oxygen in one liter of water at 30°C in equilibrium with air at one atmospheric pressure is normally 1.48×10^{19} molecules/liter but at -330 mV, this becomes 1.48×10^{-56} molecules/liter. This indicates that at such states, it is impossible to achieve the reduced state required just by removing oxygen but also it has to be reduced by adding some reduced system at a much lower potential. The reduction of media and solutions used in the isolation and culture of anaerobic bacteria had been described by Hungate as early as 1947, and since then it has undergone many changes (Bryant and Burkey 1953a; Scott and Dehority 1965).

Basically, all media and solutions used for the culture of the microorganisms are pre-reduced with cysteine-hydrochloride (cysteine-HCL). The gas (carbon dioxide or nitrogen) used during reduction is passed through a vertical column of copper filings heated to 350°C called a deoxgenator to remove traces of oxygen present in the gas. The anaerobic chamber has also evolved in prominence in the last decade (Aranki et al. 1969; Aranki and Freter 1972; Dowell 1972). There are many advantages associated with the chamber especially the dispelling of laborious preparation of large volumes of media and solutions resulting in a great reduction in preparation time (plating vs. roll tube method) but its foolproof operation and efficacy when compared with the conventional roll tube cultural method have not been well documented and should provide a large scope for investigation.

Over 2,000,000 tonnes of residues such as rice straw and palm pressed fiber (P.P.F) are produced in Malaysia every year. Palm pressed fiber is a by-product of the Malaysian palm oil industry. It is very resistant to digestion and is quite similar to fibrous straw, containing on the average 79.3% neutral detergent fiber and around 6.2% of crude protein on a dry matter basis (Jelan 1984). The estimated availability of palm oil by-products in peninsular Malaysia in 1980 was palm kernel cake (241,300), palm pressed fiber (1,304,000) and palm oil sludge (421,500) tonnes per year (Hong 1983). These agricultural by-products represent an enormous amount of resource for the production of sheep, cattle, goats and

buffaloes. The major problem in using these for the production is the need for supplements in small quantities (Preston & Leng 1984; Doyle et al. 1986) that stimulates their use by these animals. These requirements are well known and even when given, production is low to moderate (for rice straw with molasses/urea blocks, a small number of cattle will grow at about 300g/day). If an improvement in digestibility is brought about by alkaline chemicals or steam and pressure, the production is higher i.e. about 700g/day (Kategile, 1982). These approaches to increase digestibility of straw or P.P.F are not practical because of the high costs and labour requirements.

An approach which is possible is to develop more active microorganisms in the rumen which will increase the rate and extent of digestion of straw and P.P.F in the rumen. By cloning the cellulase gene into other microorganisms which might be more efficient and competitive in their ecological niche, it might be possible to bring about an increase in the overall digestion of fibrous feeds. But the possibilities of rumen manipulation lies not only in this aspect. The rumen is such a complex yet essential organ to ruminants that plans are already on the drawing board to utilize it to a greater extent.

The application of biotechnology in animal nutrition to increase the digestibility of forages lies in the establishment of three departments, each vital in its own right. The first and foremost is the basic and extensive knowledge of microbiology of

the rumen. Only with an understanding of the various species, physiology, biochemistry and nature of the microbes involved in the digestion of feeds can we only begin to proceed. Then the genetic engineers play the part of the manipulators and finally the nutritionists who will be required to monitor and trace the efficacy and maintenance of these organisms *in vivo*. But its success depends on the ability of the various departments to integrate and incorporate some form of coordination. It is also vital for the individual scientist to master and equip oneself not only with the knowledge of a particular field but to familiarize with all the interacting factors so that a more meaningful and complementary objective can be realised.

The anaerobic organism is also extensive in the medical sector with the gut bacteria implicated in the aetiology of many diseases (Abrans and Bishop 1966; Gorbach 1971; Hill and Aries 1971; Drasar and Hill 1974) like cancer of the colon and breast and even some forms of gallstones, but the autochthonous gut flora can also protect against infectious diseases and dangerous organisms. A pre-inoculation with normal gut flora could therefore help in recently de-faunated or newborn patients in the medical and veterinary sector (Savage 1980; Cheng *et al*. 1981a). Microorganisms in the gut can synthesize vitamins such as vitamin K and B-complex. These were shown to be synthesized and absorbed in the human colon as well (Hotzel and Barnes 1966; Deutsch, 1966). The advantages of G-I tract organisms are not only evident in ruminants but also in non-ruminants such as pigs which can digest up to 90% of the