CELLULOLYTIC AND ANTIMICROBIAL PROPERTIES OF SOME BACTERIA ISOLATED FROM DOMESTIC ANIMALS

[1]Kutlieva G.J., [2]Turaeva B.I., [3]Kamolova H.F, [4]Haydarov B.H.
[1,2,3]Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan
[4]Yangiyer branch of the Tashkent Institute of Chemical Technology
https://doi.org/10.5281/zenodo.10402638

Abstract. *In recent years, there has been an interest among researchers in the search for new microorganisms with probiotic properties. Among them, many studies in this regard are devoted to Bacillus subtilis, which is widespread in nature and is not pathogenic for animals and humans. Some results of a study of the biological properties and antagonistic activity of Bacillus subtilis are presented, which was carried out with the aim of developing methodological approaches to identifying strains with antagonistic activity against certain types of opportunistic microorganisms and their further use as probiotic preparations. According to cultural, morphological and biochemical characteristics, the studied bacterial strains corresponded to the species characteristics of Bacillus subtilis and were confirmed by genetic identification using the 16 S rRNA method. As a result of the experiments, the antagonistic activity of Bacillus subtilis strains against Staphylococcus aureus, Candida albicans, and E. coli was established. The growth inhibition zone of these crops ranged from 15 to 25 mm. The studied Bacillus subtilis strains can be proposed for use as probiotics.*

Keywords: *microorganisms, cellulose, probiotics, antimicrobial activity, opportunistic bacteria, carboxymethylcellulose, cellobiose.*

INTRODUCTION

There are many questions about increasing productivity, which are determined by the physiological and biological characteristics of animals. Organizing the establishment of adequate, high-quality nutrition and the creation of a new feed base for livestock farming, eliminating the causes of shortage and inferiority of feed are serious problems, the solution of which would help to increase the safety and productivity of farm animals.

A promising area of microbial biotechnology is the development of probiotic preparations for feed use. At the same time, much attention is paid to probiotics with cellulolytic properties in connection with the problems of domestic feed production. In recent years, the structure of feed raw materials in the country has undergone significant changes, which have led to the forced introduction of difficult-to-digest and low-calorie components (bran, rye, oats, barley) into feed. Of great importance is the refusal to use meat and bone meal in the feed of farm animals and replacing it with protein of plant origin (soybean meal, corn gluten), the commercial forms of which contain fiber impurities. This leads to an increase in the proportion of difficult-to-digest fiber in feed and poses the challenge of increasing its absorption, since fiber has a significant impact on the use of dietary nutrients by animals. The accumulation of plant waste enriched with fiber (brewer's grain, various types of meal, pulp, etc.) prompts attempts to utilize it by introducing it into the feed of farm animals, which also necessitates the development of drugs that stimulate the digestion of fiber [1;2]. Therefore, it is important to study the internal food chains of herbivorous animals with a high degree of digestibility of cellulose fibers, to isolate from them cellulolytic and other symbiont bacteria involved in digestion, and to develop biotechnology for the industrial production and use of such microorganisms. Cereal crops (oats, rye, barley, wheat, etc.) are widely used for the production of feed used in livestock farming. However, these nutrient sources contain non-starch polysaccharides (NSPs), which negatively affect feed digestibility. The

entry of soluble NPS with feed into the gastrointestinal tract of monogastric animals (poultry) leads to the formation of viscous jelly-like substances that impede the access of digestive juices to nutrients, impairing their digestibility [2, 3, 4]. In this regard, difficulties arise in the assimilation of polysaccharides, cellulose, and feed, respectively.

Studying the characteristics of the cellulolytic activity of rumen symbiont microorganisms is necessary to improve digestion and absorption of fiber in ruminants, and is also important for the physiological basis of their nutrition [10,15,17].

The main problem is that cellulose is very resistant to various influences. Therefore, there is a constant search for new strains of microorganisms with a higher level of cellulase biosynthesis, and biotechnological methods for using cellulose and, first of all, cellulose-containing crop waste and organic fertilizer are being developed. Such developments are impossible without the search for new strains that produce cellulolytic enzymes [16,18]. Our research is aimed at searching for bacteria with cellulolytic activity from the rumen of some domestic animals (rabbits, domestic goats and birds), and some insects. So, the purpose of this work is to isolate microorganisms-bacteria from the rumen of animals and select strains with cellulolytic activity.

More than 20 isolates of various bacteria have been isolated from animals. Opportunistic bacteria were excluded from the list of bacteria studied. The dominant group among all isolated microorganisms are bacteria of the genus Bacillus. The selected strains were identified as representatives of the species Bacillus subtilis, Bacillus megaterium, Bacillus pumilis. To identify the species of microorganisms, a Brucker MicroFlex LR MALDI-TOF mass spectrometer and specialized Maldi Biotyper software (Brucker) were used. These bacteria were identified using molecular genetic analysis of the nucleotide sequence of the 16S ribosomal RNA gene and their harmlessness was determined by the State Veterinary Service of the Republic of Uzbekistan [19]. Cellulolytic bacteria of the genus Bacillus, which are an important link in the carbon cycle in nature and an essential part of the ecosystem, are of great interest. In this regard, it seems promising to study the possibility of using them as a basis for obtaining a new producer of cellulolytic enzymes. Previously, we conducted studies to determine the endogluconase activity of local termites Anacanthotermes turkestanicus; strains isolated from insects showed more active cellulase activity than animal bacteria [20].

Research methods. The work used strains of microorganisms capable of biodegrading cellulose, isolated from the rumen of domestic animals.

Cultivation of bacteria was carried out in flasks on rocking chairs for 2 days at a temperature of 37°C in MPB (meat-peptone broth) medium containing: meat extract, dry enzymatic peptone, sodium chloride. 0.5% sodium salt of carboxymethylcellulose (Na-CMC) and cellobiose were added as a carbon source. The formation and activity of enzymes of the cellulase complex were assessed by their effect on substrates: on Na-CMC - endoglucanases, on cellobiose - cellobiases (β-glucosidases). Screening for bacterial activity was carried out in two stages. The first stage consisted of direct selection of cultures of various bacterial species from their inoculations on the surface of an agar medium with various cellulose-containing substrates as a carbon source. Based on the diameter of the color clearing zones around the grown colonies after staining the dishes with Congo red dye, the activity of enzymes produced by the cultures was judged [11,13]. The activity of enzymes in strains selected as a result of primary screening was assessed by the ability to hydrolyze soluble, medium-viscosity carboxymethylcellulose and cellobiose.

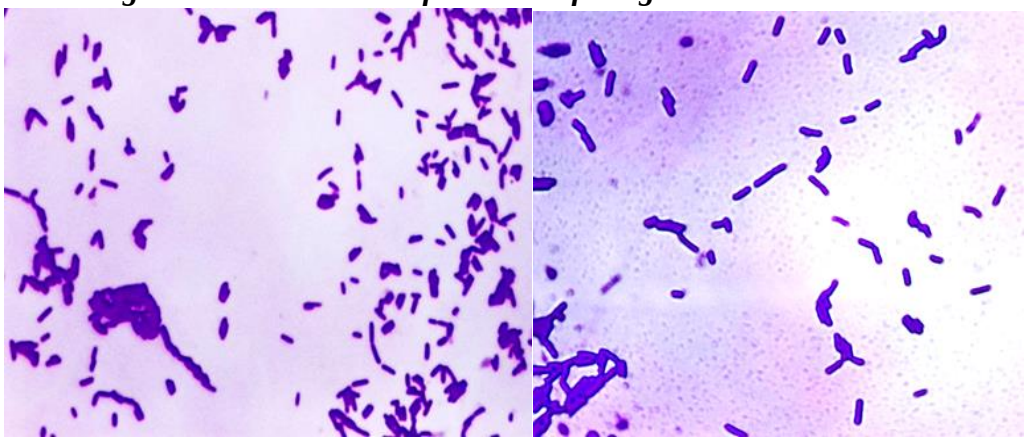
Determination of total reducing sugars (TS) Among the various methods for the quantitative determination of TS, the Somogyi-Nelson method and the 3,4-dinitrosalicylic acid (DPS) method are most widely used. Our studies used the Somogyi-Nelson method. [5,7,14].

Results and its discussion.

Isolation of strains of microorganisms promising for use as producers of cellulolytic enzymes from the rumen of domestic animals was carried out at the Department of Animal Physiology of Samarkand University. Cultures of microorganisms were isolated by seeding directly from the submitted samples. Liquid media were used as accumulation media for isolating microorganisms. Strains were selected for their ability to hydrolyze CM cellulose and cellobiose.

As a result, 10 bacterial strains exhibiting cellulase activity were isolated. Based on the data obtained from the study of the physiological and biochemical properties of isolated cellulolytic microorganisms [12], according to Bergey's determinant [13], 6 strains based on a set of characteristics were identified as representatives of the genus Bacillus (gram-positive straight rods forming endospores, motile, aerobes or facultative anaerobes, 2 catalase-positive cultures), which were selected for further work. The selection of strains of spore-forming bacteria as potential producers of cellulases when sowing cultures on the surface of agar media corresponding in composition showed that cultures that are capable of forming active cellulases gave clearing zones around the colonies, clearly visible after staining with a dye (Fig. 1-3).

Fig.1. Isolated cultures of bacteria of the genus Bacillus subtilis



A ***B***

Fig.2. Isolated strains of bacteria from the gastric juice of a domestic goat Bacillus megaterium (A); Bacillus pumilis (B).

K (control) - there is no zone, cups with clarification show cellulose hydrolysis.

Starch hydrolysis was studied on potato peptone agar. Petri dishes with seeded agar were filled with Lugol's solution after 48 hours of incubation at 37°C. Light zones around the crops indicated starch hydrolysis.

Methods for testing sensitivity to antibiotics. (Disk method) [21].

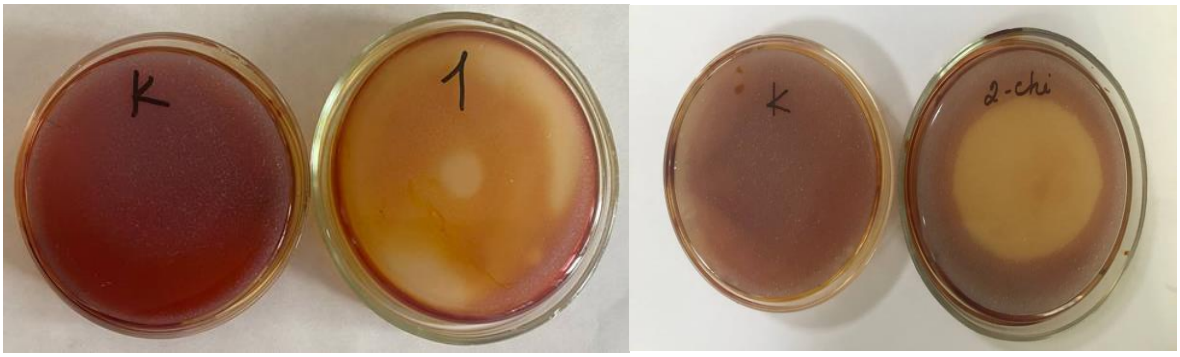


Fig. 3. Zones of hydrolysis of Na-CMC by bacilli cultures.

The sensitivity of the isolated bacteria to various antibiotics was determined: rifampicin, ofloxacin, cefotaxin, ampicillin, azithromycin, erythromycin, tetracycline, gentamicin, chloramphenicol, ciprofloxacin. Antibiotic sensitivity testing allows us to determine the resistance and sensitivity of microorganisms to drugs. Special disks of filter paper, which are impregnated with various antibiotic solutions, are placed on top of colonies of microorganisms on a nutrient medium. The presence or absence of bacterial growth reveals the degree of sensitivity to a spectrum of antibiotics.

Table 1.

Antibiotiklar	Bacillus subtilis-1	Bacillus subtilis-2	Bacillus subtilis-3	Bacillus subtilis-4	Bacillus megaterium	Bacillus pumilis
Rifampicin	16	20	4	10	7	9
Ofloxacin	30	30	26	26	22	20
Cefotaxime	0	0	0	0	0	0
Ampicillin	0	0	0	0	0	0
Azithromycin	15	12	10	25	25	
Erythromycin	0	0	0	0	0	0
Tetracycline	14	18	8	10	14	10
Gentamicin	0	0	0	0	24	26
Chloramphenicol	25	20	25	25	25	
ciprofloxacin	32	32	28	0	24	22

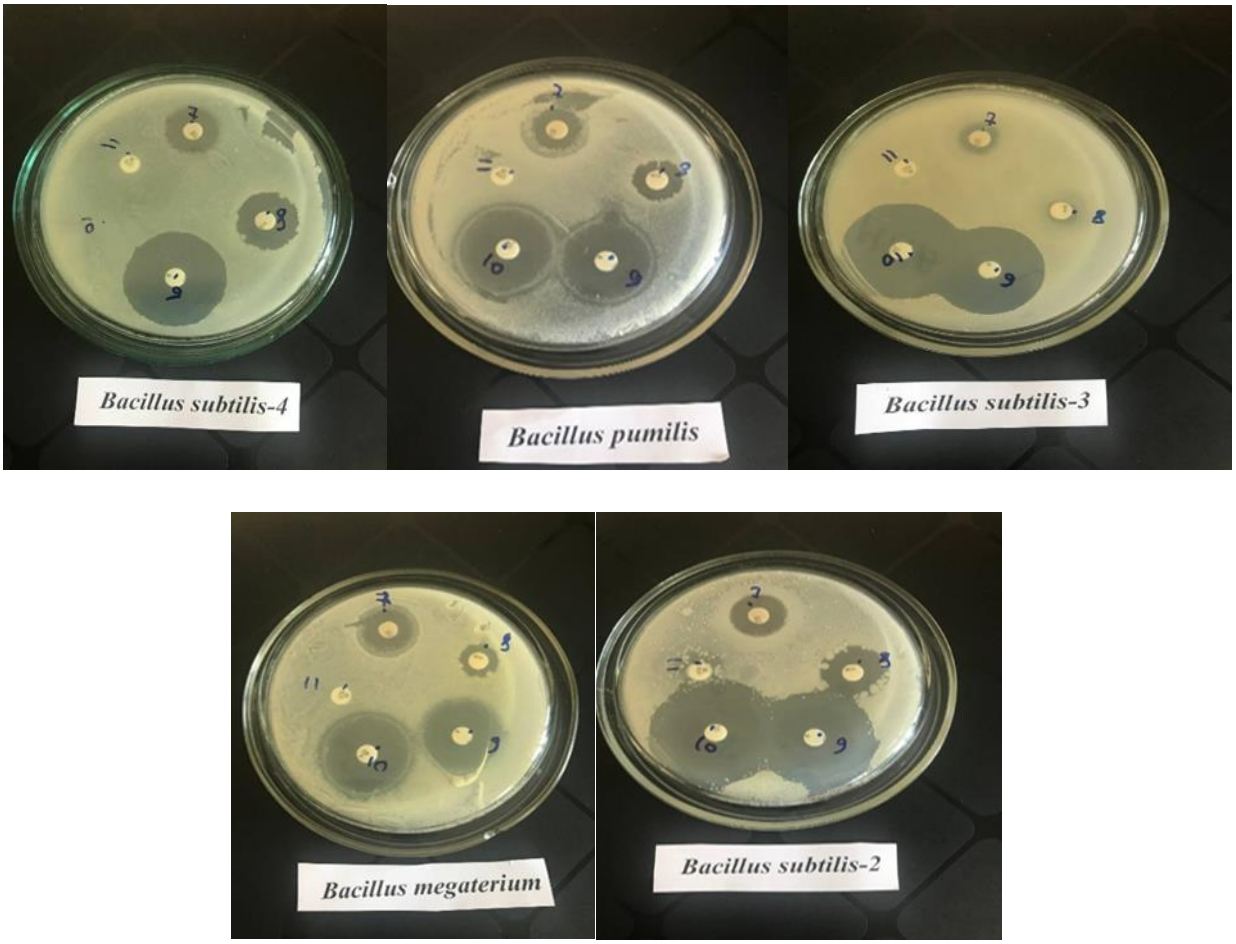


Fig. 4. Determination of sensitivity to antibiotics. (Disk method).

Antibiotic susceptibility studies have shown high sensitivity to ofloxacin, chloramphenicol and ciprofloxacin. (Table 1.) The zone of growth inhibition ranged from 25 to 32 mm. It should be noted that all the studied cultures showed resistance to the antibiotics cefotaxin, ampicillin, erythromycin and gentamicin. Bacillus megaterium turned out to be sensitive to gentamicin. The sensitivity zone was 24 mm.

Primary screening showed that in most of the studied strains, hydrolyzing soluble CMC, they have the ability to form a complex of extracellular cellulases. Thus, strains of the species Bacillus subtilis, Bacillus megaterium, Bacillus pumilis had the ability to form extracellular enzymes that break down Na-CMC.

Methods for determining the antimicrobial effect of some cellulolytic strains.

Antimicrobial activity was studied using the droplet method [22]. For this purpose, spore cellulolytic bacteria were incubated in MPB broth for 48 hours. A 5 µl sample of these bacteria was then dropped onto soft Mueller-Hinton agar. The inoculated plates were left at room temperature for 30 minutes and incubated at 37°C for 48 hours. The experiments used opportunistic microorganisms stored in the collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan, as well as kindly provided by the Department of Microbiology, Immunology and Molecular Genetics of the Center for the Development of Professional Qualifications of Medical Workers. Selected opportunistic bacterial strains were cultivated on MPA media, and yeast of the genus Candida on Sabouraud media. After incubation, isolates grown in broth at 37°C for 48 hours were adjusted to 0.5 McFarland turbidity in 0.85% saline. After thorough mixing, 7 ml of soft agar was slowly poured onto the surface of Petri dishes inoculated with spore bacteria. After cooling the agar, the Petri dishes were incubated at 28-30°C.

After incubation, the diameters of the zones of grown colonies were measured. The study was carried out in 2 replicates.

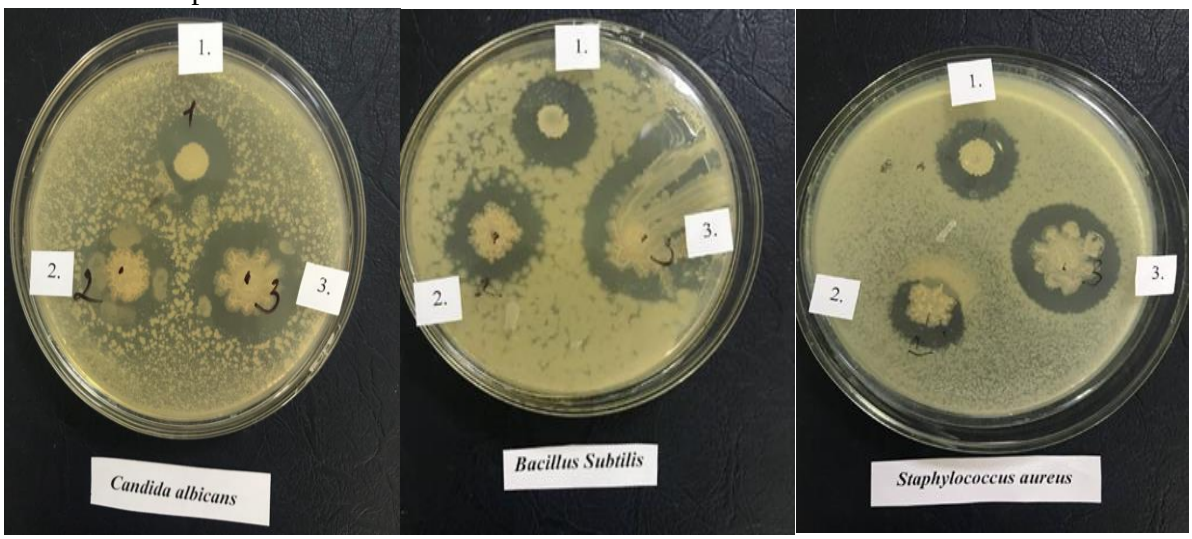


Fig. 5. Determination of the antimicrobial activity of some isolated strains of cellulolytic bacteria of the genus Bacillus subtilis, Bacillus pumilis (diameter zones of antimicrobial action are indicated in mm)

Table 2.

Isolated strains	*Staphylococcus aureus*	*Candida albicans*	*Bacillus subtilis*	*E.coli*
Bacillus subtilis-(термит)	17мм	20мм	25мм	15мм
Bacillus pumilis -(домашняя коза)	15мм	15мм	23мм	12мм
Bacillus subtilis-(кролик)	25мм	22мм	25мм	17мм

As a result of the experiments, the antagonistic activity of Bacillus subtilis strains against Staphylococcus aureus, Candida albicans, and E. coli was established. The growth inhibition zone of these crops ranged from 15 to 25 mm. The studied strains of the genus Bacillus can be proposed for use as probiotics for animals.

Cellulase activity was determined by a calorimetric method based on the determination of reducing sugars (RS) formed by the action of enzymes of the cellulolytic complex on the substrate - Na-CMC and cellobiose. The method is based on the quantitative determination of reducing sugars formed as a result of the action of the enzyme cellulase on the substrate sodium salt of carboxymethylcellulose (Na-CMC), cellobiose, at a temperature of 50 °C. The amount of reducing sugars was determined using the Somogyi-Nelson method [6-9].

Calibration curve for determining the amount of reducing sugars

Calculations are performed using a calibration curve. When creating a calibration curve, D (optical density) values are entered on the X-axis and concentration values are entered on the Y-axis. The absorbance of the samples was measured on a Shimadzu UV-1800 spectrophotometer at wavelength A = 610 nm. The results obtained were processed using Excel. The arithmetic mean (M), standard deviation (±m) and statistical significance (R) were examined. Results less than $R<0.05$ were considered statistically significant.

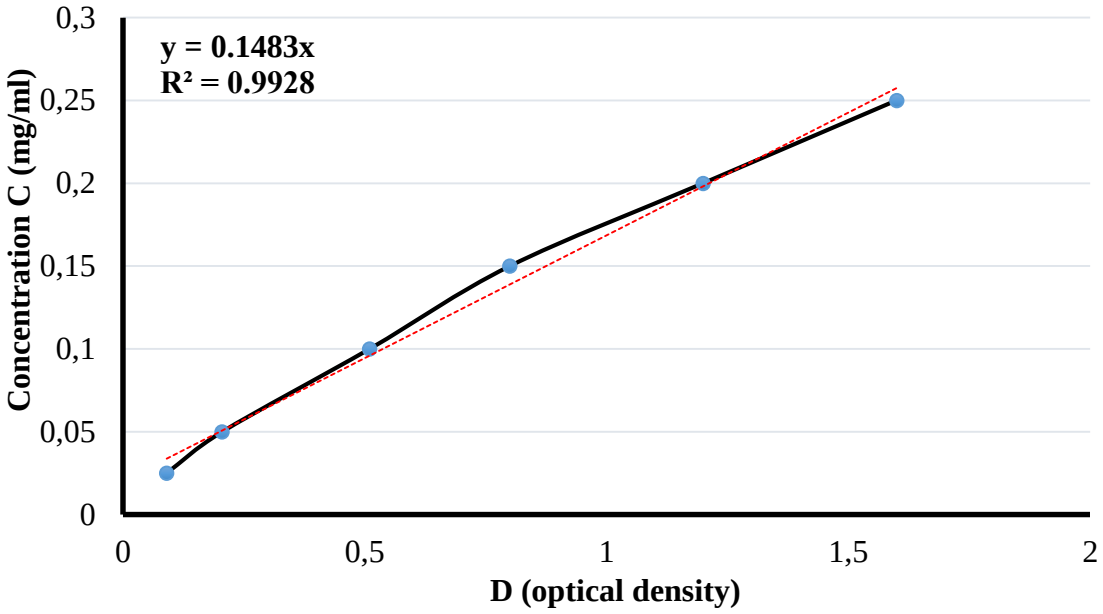
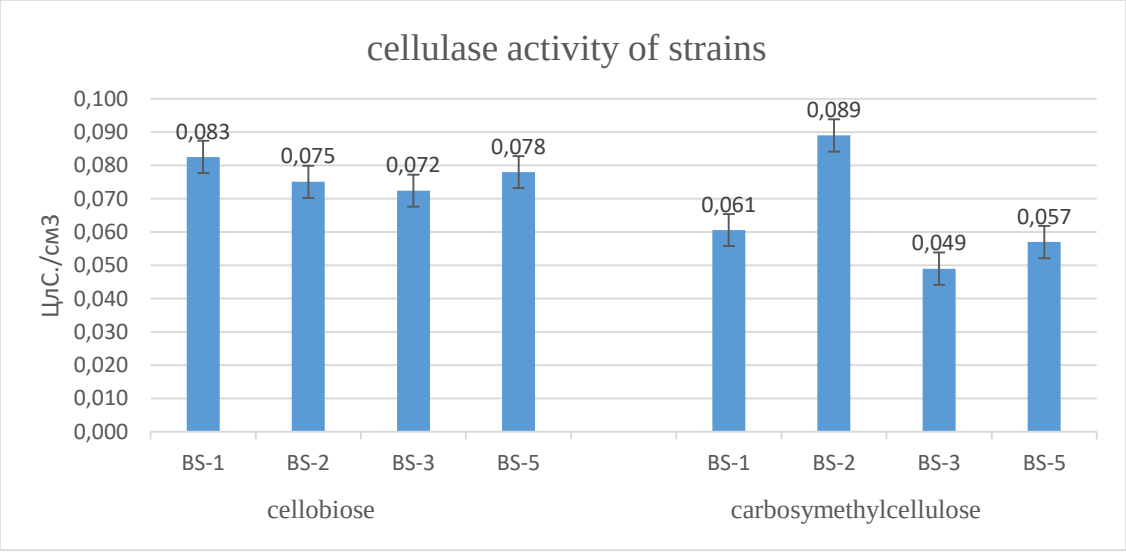


Table 3.

Experienced options, samples №	Cellulase activity (ClC/cm3)
(cellobiose)	
BS-1	0,083±0,003
BS-2	0,075±0,002
BS-3	0,072±0,003
BS-4	0,078±0,0025
(carboxymethylcellulose)	
BS-1	0,061±0,0016
BS-2	0,089±0,001
BS-3	0,049±0,0013
BS-4	0,057±0,0027



CONCLUSIONS

Studies determining the degree of sensitivity of the studied cellulolytic strains to a range of antibiotics showed high sensitivity to ofloxacin, chloramphenicol and ciprofloxacin. The growth inhibition zone is up to 32 mm. It should be noted that all the studied cultures showed resistance to the antibiotics cefotaxin, ampicillin, erythromycin and gentamicin. This indicates that bacteria isolated from domestic animals are resistant to these antibiotics. These antibiotics cannot be used to treat animals.

The first stage of screening showed that the majority of the studied strains hydrolyze soluble CMC and cellobiose. Most of the studied bacilli strains synthesized endoglucanase and cellobiase. Culture No. 2, which was isolated from the rumen of a domestic goat, had the highest synthesis of endoglucanase. Endoglucanase activity was 0.089±0.001(ClC/cm3). All cultures had the highest synthesis of cellobiase. Strain No. 1 has a β-glucosidase synthesis of 0.083±0.00 (CLS/cm3) (Table 2). Cellulase capacity (ClC) is calculated in the analyzed sample in units of ClC/g or units of ClC/cm3.

Thus, as a result of screening studies, 4 strains of bacteria of the genus Bacillus belonging to Bacillus subtilis, Bacillus megaterium, Bacillus pumilis have endoglucanase and cellobiase activity. When determining the cellulase activity of cultures, all studied strains had the highest synthesis of β-glucosidase. All studied cultures also have endoglucanase activity. The studies carried out confirmed the presence of endoglucanase and cellobiase activity in all 4 selected bacterial cultures. These strains can be used in further biotechnological research as part of biological products used as a feed additive for farm animals and for the treatment of organic and plant waste. Further research is planned using these bacteria in feed additives to determine some of the physiological processes of digestion in domestic animals.

REFERENCES

1. Bach A. Ruminant nutrition symposium: Optimizing performance of the offspring: Nourishing and managing the dam and postnatal calf for optimal lactation, reproduction, and immunity J. Anim. Sci., 90 (2012), pp. 1835-1845, https://doi.org/10.2527/jas.2011-4516
2. Bedford, M., and Partridge, G. (2010) Enzymes in farm animal nutrition, CABI, UK.
3. Bacic, A., Fincher, G., and Stone, B. (2009) Chemistry, Biochemistry and Biology of (1–3) β-Glucans and Related Polysaccharides, Academic Press, N.Y
4. J. Laporta, F.C. Ferreira, V. Ouellet, B. Dado-Senn, A.K. Learn more about the role of rumen microorganisms and their impact on the host's performance and health. Journal of Dairy Science (https://www.sciencedirect.com/journal/journal-of-dairy-science) Volume 103, Issue 8 (https://www.sciencedirect.com/journal/journal-of-dairy-science/vol/103/issue/8), August 2020, Pages 7555-7568.
5. Безбородов А.М., Астапович Н.И. Секреция ферментов у микроорганизмов. – Москва: Наука, 1984. – 72 с.
6. Билай В.И. Пидопличко Н.М., Тарадий Г.В. Целлюлолитические свойства плесневых грибов и принципы отбора активных продуцентов целлюлаз // Ферментное расщепление целлюлозы. – М.: Наука, 1967. – С. 37–45.
7. Биссвангер Х. Практическая энзимология, М., ЮИНОМ. Лаборатория знаний. 2010
8. Полыгалина Г.В., Чередниченко В.С., Римарева Л.В. Определение активности ферментов. Справочник. М., ДеЛи принт, 2003
9. Синицын А.П., Черноглазов В.М., Гусаков А.В. Методы изучения и свойства целлюлолитических ферментов. Итоги науки и техники, сер. Биотехнология. ВИНИТИ, т.25, 1993

10. Кащаев А., Петренко А., Калашников А. Кормовые добавки на основе живых культур микроорганизмов // Птицеводство. – 2006. – № 11. – С. 43-45.
11. Клесов А.А., Рабинович М.Л. Ферментативный гидролиз целлюлозы // Биологическая химия. Итоги науки и техники. – Москва, 1982. – Т. 8, № 11. – С. 1490–1496.
12. Преображенский С.Н. Фармакодинамические основы и перспективы применения ферментных препаратов в животноводстве // Ветер. с.х. жив. – 2006. – № 1. – С. 71–75.
13. Рабинович М.Л., Черноглазов В.М. Клесов А.А. Классификация целлюлаз, их распространенность, множественные формы и механизмы действия целлюлаз // В сб.”Итоги науки и техники ВИНИТИ”. Биотехнология. – 1988. – Т. 11. – С. 1–224.
14. Рухлядева А.П., Полыгалина Г.В. Методы определения активности гидролитических ферментов. – Москва: Легк. и пищ. промышленность, 1981. – 288 с. 48 ISSN 0201-8462. Мікробіол. журн., 2009, Т. 71, № 5
15. Рядчиков В., Петренко А., Радуль А. Бацелл в кормах для кур и ремонтного молодняка // Птицеводство. – 2005. – № 1. – С. 23–24.
16. Синицын А.П., Митькевич О.В., Калюжный С.В., Клесов А.А. Изучение синергизма в действии ферментов целлюлазного комплекса // Биотехнология. – 1987. – № 1. – С. 39–46.
17. Ушакова Н.А., Белов Л.П., Варшавский А.А., Козлова А.А., Колганова Т.В., Булыгина Е.С., Турова Т.П. Расщепление целлюлозы при дефиците азота бактериями, выделенными из кишечника растительноядных позвоночных // Микробиол. журн. – 2003. – 72, № 3. – С. 400–406.
18. Fumiyasu F.,Toshiaki K., Koki H. Purification and Properties of a Cellulase from Alkalophilic Bacillus sp. № 1139 // J.of Gen. Microbiol. – 1985. – 131. – P. 3339-3345.
19. Bakhora Turaeva, Guzal Kutlieva, Khulkar Kamolova, Nigora Zukhritdinova. Isolation and identification of bacteria with cellulolytic activity from the microflora of animal juice //Journal of Wildlife and Biodiversity. -2023. -V. 7(4). -P. 241-264. http://www.wildlife-biodiversity.com/
20. Kutlieva G. J. Turaeva B.I. Kamolova H.F. Kuziev B.U. New possibilities of application of cellulolytic bacteriya BACILLUS SUBTILIS isolated from local termites anacanthotermes turkestanicus // INTERNATIONAL ASIAN CONGRESS ON CONTEMPORARY SCIENCES VIII. Aksaray, Turkiye. -2023. 328. p.
21. Решедько Г.К. Определение чувствительности к антибиотикам: методы, результаты, оценка.http://www.antibiotic.ru/rus/all/articles/absens.shtml
22. Стоянова Л.Г. ВЫДЕЛЕНИЕ И ИДЕНТИФИКАЦИЯ МОЛОЧНОКИСЛЫХ БАКТЕРИЙ LACTOCOCCUSLACTIS SUBSP. LACTIS С АНТИМИКРОБНЫМ ДЕЙСТВИЕМ. Известия ТСХА, выпуск 5, 2017, DOI 10.26897/0021-342X-2017-5-41-61