

Separation and Identification of Bovine Rumen Plant Lactobacillus

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Abstract

Original Research Article

The bovine rumen is main digestive site, but there exist complex variety of microorganisms, These microbial populations are generally influenced by the food supply, host genotype, health status, living environment, and the population effects of co-living microorganisms in the environment, which affects the utilization of feed and cattle health, so it is necessary to make clear bovine rumen microbial population. In this paper, a strain of bovine rumen lactic acid bacteria (Y-B-1) was isolated from adult bovine rumen by anaerobic isolation technique. By analyzing the morphology of bacteria, combining with sugar fermentation test, using biochemical identification tube to carry out physiological and biochemical tests of the strain. Finally, the homology analysis of 16S rRNA sequence was carried out. The results showed that the strain of Y-B-1 was *Pediococcus pentosaceus*. The biological characteristics of the strain were studied, and the optimum growth temperature was determined. The results showed that the strain grew best at 30°C, pH 5.5 pH 7.5, and salt ion concentration below 7.5.

Keywords: Bovine Rumen, Separation, Identification, Physiological and Biochemical Analysis, 16S rRNA, *Pediococcus pentosaceus*.

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INTRODUCTION

The rumen is the main place for cattle to digest food. About 75%-80% of the dry matter collected was digested in the rumen, and more than 50% of the crude fiber was digested in the rumen [1]. Rumen digestion of feed is mainly the result of microbial interaction in the rumen. The rumen of cattle is home to complex and diverse microbiota, with species diversity and interaction complexity [2]. However, these microbial populations may vary depending on the type of diet fed by the animal, the living environment of the host and the population effect between symbiotic microorganisms in the environment [3]. How to improve the fermentation efficiency of rumen microorganisms and increase the fermentation subspecies of rumen bacteria is the main research method at present, which is to isolate and identify the microorganisms in the rumen and evaluate the physiological functions [4].

Spore-free gram-positive bacteria that produce large amounts of lactic acid from fermenting carbohydrates are collectively referred to as lactobacillus. A large number of studies have shown that lactic acid bacteria can regulate the normal flora of the gastrointestinal tract of the body and maintain the microecological balance, so as to produce effects on the

nutritional state and physiological functions of the body [5]. At present, lactobacillus plantarum, lactobacillus bifidum, lactobacillus acidophilus and streptococcus faecalis are mainly used in feed additives [6]. In order to obtain lactic acid bacteria with better characteristics, this study isolated and identified lactic acid bacteria in bovine rumen and studied their biological characteristics.

MATERIAL

SAMPLES AND MEDIUM

The whole rumen of adult cattle was purchased from daqing agricultural wholesale market, jiuqu district, heilongjiang province. MRS medium and Agar-agar were purchased from Beijing auboxing biotechnology co., LTD.

The Other Major Reagent

The complete set of lactic acid bacteria biochemical identification tube and other biochemical reagents were purchased from Qingdao haibo biological technology co., LTD.

METHODS

Isolation and Purification of Strains

The purchased intact adult bovine rumen was cut open with sterilized scissors under the condition of

asepsis, the contents of the bovine rumen were discarded, and the samples were sampled in multiple inner layers. The samples were thoroughly mixed in sterile normal saline, and the supernatant was taken after resting for more than ten minutes. The supernatant was diluted in a series of proportions, and the diluent was evenly coated in the prepared MRS solid medium [7]. Anaerobic culture was conducted at a constant temperature of 37°C for 24 h. The single colony with good growth was selected, and the strains were separated by streaking to obtain the pure culture. The morphology and staining characteristics of bacteria were observed by gram staining.

Physiological and Biochemical Test

Remove the complete set of lactic acid bacteria identification tube from the box, wipe and sterilize it with alcohol cotton. Fold it forcibly in the direction opposite to the easy folding point, then insert the folded tubule into the reagent rack, dip the inoculation needle into the purified culture of bacteria and inoculate into the open tubule, and seal the tubule with paraffin after inoculation. Placed it in 37±1°C incubator for culture, and 24-36 h to observe the identification tube color and gas production changes.

PRIMER DESIGN

On the basis of the dyeing characteristics and biochemical identification, it was preliminarily determined that the isolated bacteria belonged to the genus *Lactobacillus*. According to the published rRNA gene sequence in Gen Bank, Primer Premier 5 software was used to design PCR to amplify the universal primers. The upstream Primer (F) sequence was 5'-AGAGTTTGATCMTGGCTCAG-3', and the downstream Primer (R) sequence was 5'-TACGGYTACCTTGTTACGAC TT-3. The primers were synthesized by Jilin Kumei Biotechnology Co., LTD.

Extraction and PCR amplification of bacterial genome

According to the instructions of the bacterial genome extraction kit, the bacterial genome was extracted as follows:

1 mL of freshly cultured bacterial solution was placed in a clean centrifuge tube, and the precipitate was centrifuged. Solution A and lysozyme were added to lysozyme to lyse the bacteria, and protease K was added to degrade the protein, which was passed through the column and eluted to obtain genomic DNA.

The obtained bacterial DNA was used as the template and the designed F and R primers were used to amplify the 16S rRNA gene of y-b-1 strain. The amplified PCR product was purified and sequenced by Jilin Kumei Biotechnology Co., LTD.

16S rRNA Sequence Analysis

The homology of the sequencing results was compared with those of lactic acid bacteria of the same and different genera in Gen Bank by BLAST software.

Analysis of Strain Growth Characteristics Determination of Optimum Growth Temperature of Strain

The isolated and purified single colony was inoculated into MRS liquid medium for overnight culture, and then was inoculated into the new MRS liquid medium with 2% inoculum. The bacteria were placed at 16°C, 23°C, 30°C, 37°C, 44°C, 51°C and 58°C, respectively, for anaerobic culture. The OD600 value of the bacterial liquid was determined 24 h later and the curve was drawn.

Determination of Strain Growth Curve

After purification, pick a single colony vaccination to MRS liquid medium, 30°C under anaerobic conditions for the night, and then use liquid move from 100 (including 1 microbial inoculation to 5 mL new MRS liquid medium, 30°C anaerobic conditions continue to develop, sampling once every 2 h, shake to determine microbial OD600 value, draw the bacteria growth curve after 24 h.

The acid resistance of the strain was determined

The purified bacterial solution was inoculated into MRS liquid medium with initial pH of pH 1.5, pH 3.5, pH 5.5, pH 7.5, pH 9.5 and pH 11.5, respectively. After anaerobic culture at 30°C for 24 h, the OD600 value of bacterial solution of different pH was shaken and sampled to determine the optimal pH value of the strain.

The Salt Tolerance of the Strain Was Determined

According to the above proportion, the purified bacterial liquid was inoculated into MRS liquid medium with concentration of 1.5%, 3.5%, 5.5%, 7.5%, 9.5% and 11.5% NaCl, respectively. After anaerobic culture at 30°C for 24 h, the OD600 value of bacterial liquid of different pH was shaken and sampled to determine the optimal salt concentration of bacterial strain.

RESULT

Observation of Colony and Body Morphology

Strains were isolated from bovine rumen and cultured using MRS solid medium under anaerobic conditions for 24 h. It could be seen that the colony of y-b-1 strain was milky white with smooth surface and protruding on the surface of the medium. The colony size was different and opaque, with a diameter of 1 ~ 2 mm (Figure-1 and Table-1).

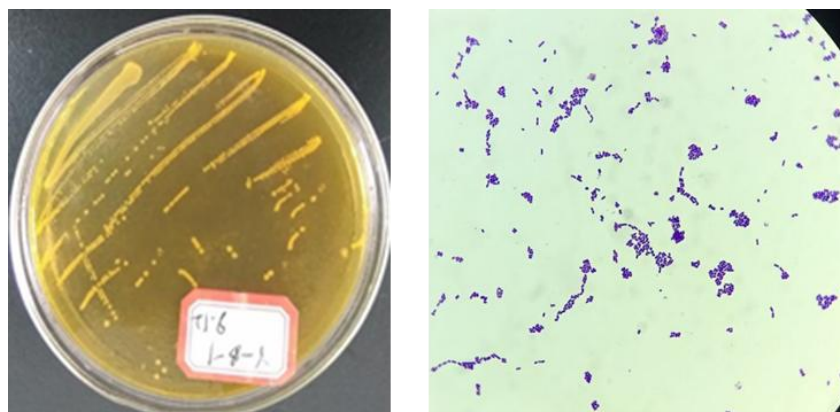


Fig-1: y-b-1 colony and oil mirror

Table-1: Gram staining and morphological observation of colony morphology

Strain	Gram staining	Colony characteristics	Colony morphology
Y-B-1	G+	The growth rate was fast on MRS plate, and the colonies were milky white, smooth and round on the surface, protruding on the surface of the medium. The colonies varied in size, with neat edges and opaque edges. The diameter was 1 ~ 2 mm.	The growth along the puncture line of the vegetable was round like a filamentous cell or round with obtuse at both ends, without spores or flagella.

Sugar Fermentation Test

Single colonies were selected and inoculated into the biochemical tube, sealed with sealing membrane, and incubated at 37°C for 36 h. The results were obtained by comparing colors. According to the

sugar fermentation test, this strain can ferment heptanoside, fibredisaccharide, maltose, mannitol, salicylate, sorbitol, sucrose, raffinose, inulin, lactose and 1% sodium hippuric acid (Table-2).

Table-2: Physiological and biochemical identification results of the strain

Biochemical identification tube for lactic acid bacteriao	Y-A-2
Seven leaf glycoside	—
Fiber two sugar	+
Malt dust	—
Mannitol	—
Salicin	+
sorbitol	—
saccharose	—
gossypose	—
synanthrin	+
milk sugar	—
1% sodium mauric acid	—

16S rRNA Sequence Analysis

The general primers for PCR amplification of 16S rRNA gene fragment were designed. Jilin kumei Biotechnology Co., Ltd. was entrusted to synthesize the primers and detect the sequences, and blast was used for sequence comparison. The results showed that the isolated strain Y-B-1 had 2330 nucleotide sequences, which were 100% homologous with *pediocus acidilactici* strain hxbm 408 16S ribosomal RNA gene,

partial sequence ID: mf992210.1, etc. published in GenBank. The specific comparison results are shown in Table-3.

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Table-3: Comparison of 16SrRNA homology with a variety of pentoses

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pediococcus pentosaceus gene for 16S ribosomal RNA, partial sequence, strain: ZZU A22	2330	2330	100%	0	99%	LC119127.1
Pediococcus pentosaceus strain ON-81A 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KX527659.1
Pediococcus pentosaceus gene for 16S ribosomal RNA, partial sequence, strain: ZZU 64	2330	2330	100%	0	99%	AB831184.1
Pediococcus pentosaceus strain wg8 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	JX477168.1
Pediococcus pentosaceus strain MRSF2 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KF048926.1
Pediococcus pentosaceus strain PP 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	HQ286591.1
Pediococcus pentosaceus gene for 16S ribosomal RNA, partial sequence, strain: MJK7	2330	2330	100%	0	99%	AB494722.1
Pediococcus pentosaceus strain CAU85 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	MF424141.1
Pediococcus pentosaceus strain SP-024 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KX527660.1
Pediococcus pentosaceus strain ON-89A 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KX387352.1
Pediococcus pentosaceus strain SNR-1 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KU728709.1
Pediococcus pentosaceus strain ZTR-2 16S ribosomal RNA gene, partial sequence	2330	2330	100%	0	99%	KU728706.1

Analysis of Strain Growth Characteristics

Determination of Optimum Growth Temperature of the Strain

The activated lactobacillus was inoculated to the inoculation dose at 2%. The activated lactobacillus pentococcus was inoculated to MRS liquid medium at 2% inoculation dose, and then was cultured at 16°C, 23°C, 30°C, 37°C, 44°C, 51°C, and 58°C, respectively. It can be seen from the respectively. It can be seen from the figure2 that the OD600 value of this strain is relatively low when the temperature is lower than 23°C and higher than 37°C, while the OD600 value is the highest when the temperature is 30°C, indicating that the strain grows best at 30°C, grows slowly at other temperatures, and almost stops growing when the temperature is higher than 58°C.

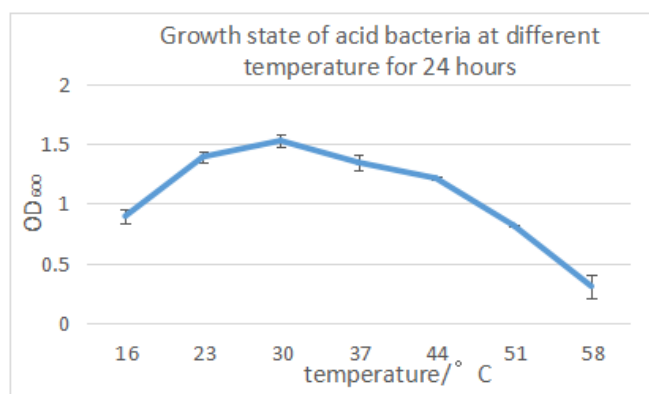


Fig-2: Y-B-1 at different temperatures

Determination of Strain Growth Curve

With 2% inoculation amount will activate the amount of lactic acid bacteria to inoculated will activate pediococcus pentosaceus to 2% inoculation amount to MRS liquid medium, at 30°C under the condition of anaerobic culture for 24 h, take a sample a defective goods every 2 h, shake to determine microbial OD600 value, draw the growth curve of lactic acid bacteria after 24 h, the result is visible as the extension of time strain concentration was gradually rising trend, and strains belong to the logarithmic phase, after 16 h almost close to a plateau, the strain growth characteristics and conventional bacterial growth trend, the specific results as shown in Figure-3.

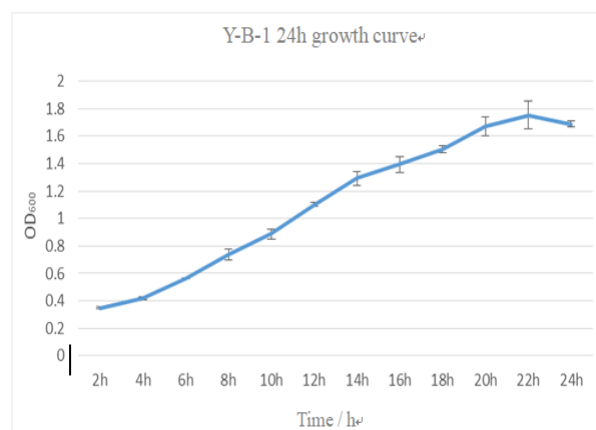


Fig-3: Y-B-1 24h growth curve

Determination of acid resistance of the strain

With 2% quantity of activation of four strains of lactic acid bacteria inoculated to initial inoculation amount will activate *Pediococcus pentosaceus* to 2% inoculation amount to the initial pH 1.5, respectively, pH 3.5, pH 5.5, pH 7.5, pH 9.5, pH 11.5 MRS liquid medium, at 30°C under the condition of anaerobic culture, 24 h after the shake sampling determination of different pH gas-bacilli OD600 value, and draw curves as shown in figure 5, the figure shown strains pH less than 3.5 or pH greater than 9.5 within the scope of almost stopped growing. It grows very well in the pH 5.5 to 7.5 range.

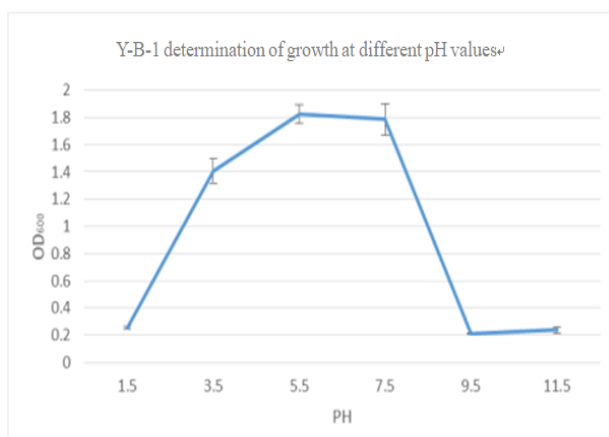


Fig-4: Y-B-1 determination of growth at different pH values

Determination of Salt Tolerance of the Strain

With 2% quantity of activation of four strains of lactic acid bacteria inoculated to initial inoculation content will activate *Pediococcus pentosaceus* to 2% inoculation amount to initial to contain containing NaCl was 1.5%, 3.5%, 5.5%, 7.5%, 9.5%, 11.5% NaCl MRS liquid medium, in 30°C under the condition of anaerobic culture, 24 h after the shake sampling determination of different pH gas-bacilli OD600 value, and draw curves as shown in figure 5, the figure shown strain can grow well under salt ion concentration is 7.5, and the salt ion is higher than 9.5 under the environment of almost stops growing.

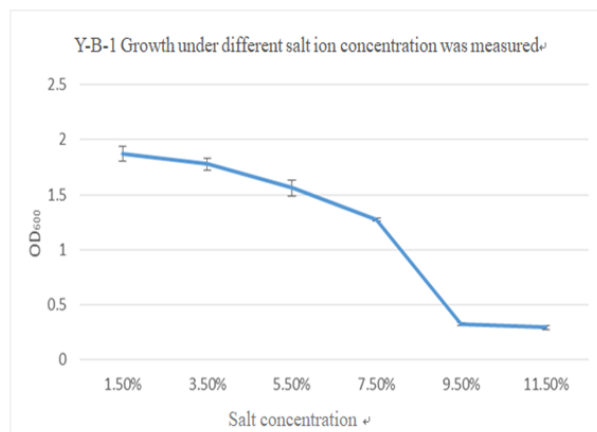


Fig-5: Y-B-1 Growth under different salt ion concentration was measured

DISCUSSION

In order for lactic acid bacteria to play a stable physiological and biochemical role in the intestine, they must enter the stomach and intestines and maintain good activity in an environment containing a variety of enzymes and low pH values. Microorganisms in the animal digestive tract have specificity and diversity, and there is a close relationship between animals and various intestinal microorganisms as well as a variety of microorganisms that are mutually utilized, selected and interdependent [8]. This experiment was isolated from adult bovine rumen Y-B-1 plant aureus *Pediococcus pentosaceus*, derived from adult bovine rumen, derived from the digestive tract, non-toxic side effects, resistance to acid enzymatic resistance is strong, can grow in the stability of the bovine rumen, easier to play a role of engraftment live from animal intestine, help probiotic feed additives and animal husbandry research [9].

In this study, Y-B-1 strain isolated from the rumen of adult cattle was identified by morphological analysis of bacteria, physiological and biochemical characteristics detection, and homology analysis of 16S rRNA sequence. The results showed that Y-B-1 biological activities such as fermentation maltose, lactose and inulin.

After the rumen contents reached a relatively stable state, the temperature was about 38-41°C. Influenced by the type of feed and the temperature of drinking water, it affects the digestion of feed food. But we separated strains grow best under 30°C temperature, instructions will be in the rumen bacteria isolated and alone in the case of many bacteria interactions of different shape instructions will be in the rumen bacteria isolated and alone in the case of many bacteria interactions of characters are different. The pH value of rumen contents was determined by volatile fatty acids in bovine rumen chyme, buffer salts in saliva and rumen epithelium to volatile fatty acids. Generally, the pH value ranges from 6 to 7. The strains isolated in this study all grew well in the range of pH 5.5 to pH 7.5, which was basically similar to the pH in the rumen environment and consistent with the growth characteristics of the bacteria in the rumen.

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