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ISOLATION and CHARACTERIZATION of STARCH-UTILIZING LACTIC ACID BACTERIA

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Lactic acid bacteria were isolated from various plants, fodder, food products as well as from rumen, intestines and excreta of calves, cows, goats, horses, pigs to reveal cultures able to digest starch. More than 600 strains were obtained and grouped according to their morphological, physiological and biochemical patterns. Preliminary identification of selected strains showed a taxonomic variety of amylolytic lactic acid bacteria within the genera *Streptococcus, Pediococcus, Enterococcus, Lactobacillus*. It was established that active strains possessing ability to utilize insoluble starch are rare and found predominantly among lactic acid bacteria – constituents of calf gastrointestinal microbial cenosis. *Lactobacillus plantarum* isolated from maize silage was selected and characterized as the most promising raw starch-utilizing strain.

Մեկուսացվել են կաթնաթթվային բակտերիաներ, որոնք ընդունակ են յուրացնել օսլա տարբեր աղբյուրներից բույսերից, սիլոսի սննդամթերքներից, ինչպես նաև գյուղատնտեսական կենդանիների (ոչխար, կով, հորթ, խոզ, ձի) աղեստամոքսային տրակտի պարունակությունից: Ստացվել և դիֆերենցվել են այդ բակտերիաների 600-ից ավելի մաքուր կուլտուրաներ իրենց մորֆոլոգիական, ֆիզիոլոգիական և կենսաքիմական պարամետրերով: Ստացված շտամների նախնական իդենտիֆիկացիան թույլ է տվել բացահայտել ամիլոլիտիկ ակտիվությամբ օժտված կաթնաթթվային բակտերիաների տաքսոնոմիական բազմազանությունը. հայտնաբերվել են *Streptococcus, Pediococcus, Enterococcus, Lactobacillus* ցեղերի ներկայացուցիչները: Հաստատվել է, որ չլուծվող օսլա յուրացնող ակտիվ շտամները հանդիպել են հազվադեպ և հաճախ մեկուսացվել են հորթերի աղեստամոքսային տրակտի միկրոբային ցենոզից: Եգիպտացորենի սիլոսի միկրոֆլորայից անջատված *Lactobacillus plantarum*-ի կուլտուրաները բնութագրվել են որպես չլուծվող օսլայի յուրացնող առավել հեռանկարային շտամներ:

Проведено выделение молочнокислых бактерий, способных утилизировать крахмал из разных источников: растений, силоса, пищевых продуктов, а также содержимого желудочно-кишечного тракта сельскохозяйственных животных (овец, коров, телят, свиней, лошадей). Получены и дифференцированы по морфологическим, физиологическим и биохимическим параметрам более 600 чистых культур этих бактерий. Предварительная идентификация полученных штаммов позволила установить таксономическое разнообразие молочнокислых бактерий, обладающих амилолитической активностью: выявлены представители родов *Streptococcus, Pediococcus, Enterococcus, Lactobacillus*. Установлено, что активные штаммы, способные утилизировать нерастворимый крахмал, встречались редко и чаще всего выделялись из микробного ценоза желудочно-кишечного тракта телят. Выделенные из микрофлоры кукурузного силоса культуры *Lactobacillus plantarum* охарактеризованы как наиболее перспективные для утилизации нерастворимого крахмала.

Key words: *Lactic acid bacteria - Lactobacillus plantarum - starch - amylolytic activity*

Lactic acid bacteria are the most strictly substrates depending microorganisms among known non-pathogenic prokaryotes. These bacteria distinguished by effective fermentative type of metabolism consume mainly mono- and disaccharides as the substrates. The ability to utilize polysaccharides, including starch, is found relatively rare in this microbial group [1]. The significance of seeking of active amylolytic strains is motivated both by possibility to reduce cultivation costs and prospects of recycling starch containing wastes, ensiling hardly fermentable plants, synthesis of end-products (lactic acid, microbial biomass) by fermentation of starch and starch-containing materials.

Lactic acid fermentation has been extensively investigated due to evident industrial applications of the product. It is widely used by the food industry (as an acidulant, as a preservative, and for stearoyl-2-lactylate synthesis), in pharmaceutical and chemical industries (for polylactic acid, green solvent, and slow release carriers). Annual global manufacture of lactic acid is close to 50,000 t, with about equal amounts being produced by fermentation and chemical synthesis [12]. Lactic acid is commercially produced by fermentation of sucrose, glucose, molasses or milk whey by *Lactobacillus sp.* A part of supply is derived from starch to decrease substrate costs by two-step process of saccharification with acid or microbial amylase followed by *Lactobacillus* fermentation [12]. Conventional biotechnological production of lactic acid from starchy materials, such as barley, maize, potato, or rice requires pretreatment, like gelatinization and liquefaction, usually at elevated temperature in limits of 90−130°C at least for15 min, followed by acid or enzymatic saccharification of starch to glucose (during 24-96 hours) and subsequent conversion of glucose to lactic acid by lactic acid fermentation [6]. Hence, application of lactic acid bacteria possessing high amylolytic activity and ability to ferment starch, starchy materials and wastes directly to lactic acid is desirable to save the time and process costs.

The main purpose of this work is isolation and characterization of lactic acid bacteria possessing a substantial amylolytic activity to obtain most promising raw starch-utilizing strains.

Materials and methods. Plant, fodder, food samples, contents of the rumen and intestines of ruminant animals as well as excreta of cows, pigs, horses, goats have been used as sources for isolation of lactic acid bacteria with amylolytic activity on media containing starch as the only carbon source. Direct plating of serial dilutions of samples on starch-containing agar as well as dilution plating after enrichment culture procedure were applied. Pure cultures were obtained from colonies showing starch lysis zones. The cultures were grown in modified MRS broth [3] containing insoluble (raw) starch as the only carbon source.

Selected bacterial isolates were examined for carbohydrate fermentation pattern and other characteristics essential for genera and species identification by standard methods [1]. Each experiment was performed at least twice to assess reproducibility, and species were identified by comparing the reaction profile with that of type strains.

Lactic acid bacteria were routinely maintained at 10°C in modified MRS medium where glucose was replaced by 1% commercial insoluble potato starch. Strains were subcultured at 20-30 - days intervals. Different methods of long-term maintenance of cultures were tested in special series of experiments. Conservation in glycerol (20-, 40-, 60-, 80% solution at -10, -40, -60°C), in liquid nitrogen (suspensions of 10% glycerol and 1% gelatin with 7% sucrose) and lyophilization

with cryoprotectors were used. 18-24- hours cultures of the third generation served as inocula (1%, v/v). The batch fermentation was conducted in 2000 ml flasks with 1800 ml of medium containing in g/l: mashed potato - 200.0, peptone - 10.0, K_2HPO_4 - 0.5, KH_2PO_4 - 0.5, $MgSO_4 \cdot 7H_2O$ - 0.2, $MnSO_4 \cdot H_2O$ - 0.05, $CaCl_2 \cdot 2H_2O$ - 0.5. The lactobacilli were cultivated at 30°C.

Viable cells (CFU/ml – colony-forming units per 1 ml of cultural liquid) were counted by final dilution technique in light (0.3 %) mash agar. Tubes were incubated at 30–37°C for 48hrs. Total titratable acidity was estimated by titration with 0.1 N NaOH in presence of phenolphthalein as an indicator. The results were expressed in Terner degrees (1°T is equivalent of 0.1 N NaOH amount (ml) sufficient for titration of 100 ml cultural liquid). Raw starch contents was controlled by measurement of dry weight after two washing and centrifugation followed by drying at 105°C for constant weight. Total starch in the media was also estimated by starch-iodine method after cell removal by centrifugation.

Amylase activity was assayed by monitoring starch degradation via measurement of its iodine-complexing ability. One enzyme activity unit was defined as the amount of enzyme sufficient for hydrolysis of 1g starch in 10 min at 40°C, pH 5.5. Lactic acid concentration was determined in the supernatant by HP 4890D unit, USA using 30m capillary column HP INNOVAX (USA) of 0.32mm internal diameter packed with fixed PEG phase 0.5μm thick. Helium served as carrier gas at flow rate 0.66ml/min. Sample volume – 1μl.

Results and discussion. *Screening, isolation and identification screening of lactic acid bacteria.* More than 600 strains of lactic acid bacteria were isolated from different sources including 200 strains from rumen, intestines and excreta of farm animals (cows, goats, horses, pigs) on media containing commercial insoluble potato starch as the only carbon source. The enrichment culture technique as well as direct plating were used. Advantage of enrichment culture technique was established. Amylolytic cultures demonstrated clear zones surrounding colonies on starch-containing media.

Obtained pure cultures were characterized by fermentative carbohydrate metabolism type with generation of acid products, absence of cytochrom-containing respiratory systems, negative catalase reaction and, in most cases, negative nitrate reductase reaction, they are air-tolerant and require nutrient factors. These properties as well as typical morphological and cultural traits (non-sporulating, non-motile gram-positive coccal- or rod-shaped cells, slight surface growth or its absence on agar media, typical colonies) allow to affiliate isolates to lactic acid bacteria.

The total titratable acidity was used as an indicator of acidogenic activity of the cultures at the initial investigation stages. Screening of active acid producers digesting insoluble starch among 400 cultures isolated from various sources (diverse plant materials, including hard-to-ensile substrates, fodder, fermented food stuffs) and 200 isolates from gastrointestinal system of young stock has revealed that active strains are relatively rare and are found predominantly among lactic acid bacteria – representatives of calf gastrointestinal microbial cenosis. The isolates differed in growth and acidogenesis on starch-containing media (active cultures were scarce). The majority of tested strains possessed low acidogenic activity (not more than 60°T) and only singular cultures generated enough acid metabolites to acidulate the cultural media over 80°T (Fig. 1). Almost twice more active amylolytic strains were isolated from rumen as compared to the intestines. Rod-shaped forms prevailed among screened cultures from different parts of gastrointestinal system: their percentage varies in terms of 59% and 73% for calf rumen and intestine isolates, respectively.

Fig.1. Acidogenic activity of lactic acid bacteria isolated from rumen (a) and intestines (b) of young stock. Titratable acidity of cultural media: 1 – below 60°T, 2 – 61-70°T, 3 – 71-80°T, 4 – over 80°T.

Preliminary identification of selected strains revealed a taxonomic variety of amylolytic lactic acid bacteria: among the strains isolated the representatives of *genera Streptococcus, Pediococcus, Enterococcus, Lactobacillus* were found. It was established that the most active strains were rods isolated from silage. Most promising strains were represented by bacteria of genus *Lactobacillus*. The majority was characterized by presence of fructose diphosphate aldolase and phosphoketolase and ability to ferment both pentose and hexose sugars. The obtained results allowed to refer these strains to facultative heterofermentative group of bacteria of the *Genus Lactobacillus*.

Acidogenic and amylolytic activity of some cultures on modified MRS medium with 1 % insoluble potato starch is illustrated in Table. The most active strain was isolated from silage and identified as *Lactobacillus plantarum*.

Table. **Growth, acidogenesis and amylolytic activity of lactic acid bacteria on starch-containing medium**

Strains	Isolation source	Biomass yield, g/l	pH	Titratable acidity, °T	Amylolytic activity, U/ml
Lactobacillus sp. 372	Calf rumen	1.34	6.1	60	0.039
Pediococcus sp. 377	Calf rumen	1.56	5.9	78	0.051
Lactobacillus sp. 378	Calf rumen	1.62	5.9	82	0.094
Streptococcus sp. 381	Calf intestines	1.48	6.1	70	0.090
Streptococcus sp. 386	Calf rumen	1.55	6.0	70	0.072
Enterococcus sp. 388	Calf rumen	2.04	5.9	89	0.103
Enterococcus sp. 390	Calf rumen	1.61	6.0	70	0.061
Lactobacillus sp. 391	Calf rumen	1.49	6.0	70	0.055
Lactobacillus sp. 354	Calf intestines	2.01	5.9	80	0.069
Lactobacillus sp. 393	Calf intestines	1.89	6.0	76	0.071
Lactobacillus plantarum	Maize silage	3.82	5.2	105	1.051

Obtained results correspond well with other reports indicating that the ability to grow and produce lactic acid on starchy substrata is a rare characteristic of lactic acid bacteria. In literature the principal species of this type are represented by *Streptococcus bovis, Streptococcus equines* [5], *Lactobacilllus amylophilus* [8,14], *Lactobacillus amylovorus* [7], *Lactobacillus acidophilus, Lactobacillus cellobiosus* [10],

Lactobacillus plantarum [4,11]. As a rule, lactic acid bacteria digest soluble or pretreated starch [6, 11, 14]. The ability to decompose raw starch and starchy substrates was demonstrated in *Str. bovis* 148 isolate from bovine rumen [9], *L. amylovorus* isolated from cattle manure corn – enrichments [13], *L. cellobiosus* isolated from municipal wastes [2] and *L. plantarum* isolated from fermented cassava [4].

Among the strains of lactic acid bacteria screened for growth and acidogenic activity, only selected isolate from silage demonstrated rather high accumulation of acid products after 24 hrs batch culture of free cells on modified MRS medium with different kinds of insoluble starch (1 %). It was found that the selected strain after 18h of growth at 30°C on modified MRS medium with 1% glucose, soluble or insoluble starch accumulated lactic acid in amounts 20.02 g/l, 7.9 g/l, 12.42 g/l, respectively.

Study on maintenance and long-term conservation of lactic acid bacteria showed that lyophilization with cryoprotectors provided hopeful viability and reproduction of strains tested up to one year. Good results were obtained with 40-60% glycerol at - 40 and - 60°C. Conservation in liquid nitrogen both with gelatin and glycerol proved more effective.

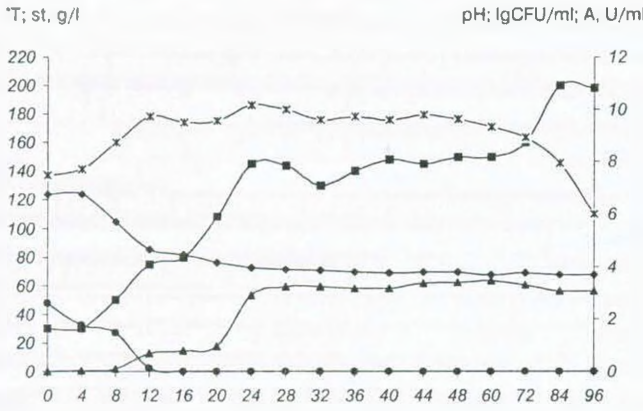

Fig. 2. Fermentation of *L.plantarum* on starchy potato medium. 1 – number of viable cells (lgCFU/ml); 2 – titratable acidity (°T); 3 – active acidity (pH); 4 – amylase activity (A, U/ml); 5 – starch (g/l)

Fermentation of selected lactobacilli on starchy potato medium. Investigation of growth, acidogenesis and amylolytic activity dynamics on starchy potato medium revealed high biological potential of the selected *L.plantarum* strain (Fig. 2). By 24 hrs of culture on medium containing 1 % of inoculum number of viable cells reached $1.5 \cdot 10^{10}$ CFU/ml. The culture selected showed effective insoluble starch transformation: by 14hrs of fermentation starch was not detected in cultural liquid. Intense accumulation of acid metabolites occurred during the growth and resulted in pH decline to 3.7 and upsurge of titratable acidity up to 200°T. Amylolytic activity increased during exponential growth phase up to 3.2 – 3.4 U/ml value by 26 – 48 h and remained at this level till the end of the culture.

Summing up the selected strain of *Lactobacillus plantarum* isolated from maize silage might be of considerable interest for industrial production of the food grade lactic acid directly from raw starchy substrates without preliminary enzymatic or chemical treatment. It may significantly reduce the costs for lactate production and process duration, facilitating solution of waste starch disposal problem.

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