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# Optimization of Cultivation Conditions for Increasing the Production of Exopolysaccharides of The Lactobacillus Plantarum Eb-2 Strain

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**ABSTRACT**

It is shown in the work that the composition of the medium and the cultivation conditions have a great influence on the biosynthesis of EPS of the *L. plantarum* EB-2 strain. It was found that the cultivation of the culture under the conditions of a shaker-incubator, in a medium based on curd whey with pH 5.5, at a fermentation temperature of 37°C, with a fermentation time of 48 hours, when using peptone and sucrose, the yield of EPS production increases to 910.3 mg/l, which is 3.6 times more than the EPS production output without selection of fermentation parameters (250.0 mg/l). The results show that the selection of the composition of the environment and the growing conditions make it possible to achieve economic efficiency in the production of new biologically active substances for the pharmaceutical and food industries.

**Key words:** Lactobacilli; exopolysaccharides; optimal cultivation conditions; sources of nitrogen; carbon.

## 1. INTRODUCTION

Among lactic acid bacteria, special attention is paid to bacteria of the genus Lactobacillus, whose representatives are widespread in nature. Various researchers have shown that lactobacilli have great potential for the synthesis of exopolysaccharides (Mecedo, 2002).

Some LABs form polysaccharides that are secreted from the cell as constituents of the cell wall (peptidoglycans). The latter are either firmly attached to the surface of the microbial cell in the form of a capsule (capsular polysaccharide, CPS) or are released into the environment as exopolysaccharides (EPS) (Chapot-Chartier *et al.,* 2011).

Microbial exopolysaccharides are the object of intensive research because of their importance in the structure and metabolism of microorganisms. Exopolysaccharides of lactic acid bacteria play a decisive role in improving the rheology, texture, taste of fermented foods, have a beneficial physiological effect on human health and have antitumor, immunomodulatory and anticarcinogenic activity (Doleyres *et al.,* 2005). LAB can also produce a variety of functional oligosaccharides. Oligosaccharides have huge industrial applications as prebiotics, nutraceuticals, sweeteners, moisturizers, drugs against colon cancer, immune stimulants, etc (Remaud-Simeon *et al.,* 2000).

The biosynthesis of exopolysaccharides is a complex chemical process that involves a large number of enzymes and regulatory proteins. Thus, in mesophilic strains of LAB, such as *Lactococcus*, the genes encoding proteins are also involved in EPS biosynthesis and are located in plasmids, while in thermophilic streptococci and lactobacilli they are located in chromosomes (Broadbent *et al.,* 2003). In the process of biosynthesis of homopolysaccharides, extracellular or cell-wall-bound glucanosaccharides are involved, using sucrose as a substrate. Glycosyltransferases transport glucopyranosyl groups from sucrose to acceptor molecules to form glycosidic bonds. The mechanism of biosynthesis of heteropolysaccharides is more complex than the mechanism of biosynthesis of homopolysaccharides. Repeating units of heteropolysaccharides are synthesized in the cytoplasm from sugar nucleotides. They are translated by glycosyltransferases across the cell membrane, where polymerization occurs by sequential addition of sugar nucleotide units to the growing chain attached to the carrier lipid, and finally, EPS is released into the medium (Patel *et al.,* 2012).

Various strains of *Lactobacillus* spp. differ in a unique way of biosynthesis of exopolysaccharides, using various carbohydrates such as glucose, sucrose, galactose, lactose, added to the growing medium. Special clusters of genes located in the chromosome of bacteria are stable during long-term development on such a food matrix (Siezen *et al.,* 2010). There are many discussions about the formation of EPS under the influence of various cultivation conditions. Cultivation conditions such as pH, temperature, composition of the growing medium and incubation time have a significant impact on the yield and composition of EPS. It has been shown that lactobacilli strains, depending on the carbon source of the nutrient medium, can produce EPS with different rheological properties (Polak-Berecka *et al.,* 2013).

Typically, the production yield of heteropolysaccharides ranges from 0.05 to 0.60 g / L (Ruas-Madiedo *et al.,* 2005). while homopolysaccharides are synthesized in large quantities: up to several grams per liter (Miao *et al.,* 2014; Wolter *et al.,* 2014; Malang *et al.,* 2015). MRS broth is the most suitable medium for the growth and synthesis of IBP biopolymers. But for industrial purposes and from an economic point of view, it is more expedient to use waste from other industries, as the basis for a nutrient medium. Tofu whey, a waste from soy cheese production, has low industrial potential due to its high-water content. However, it contains significant amounts of soluble solids (Botelho-Cunha *et al.,* 2010). This product can be used as a substrate for the cultivation of *L. plantarum* and the synthesis of metabolites such as EPS.

Many authors propose whey as the basis of the medium for increasing the production of EPS. Bukola Adebayo-Tayo (2008) describes that the composition of the medium has a profound and significant influence on the production of EPS by LAB isolates. In a comparative study of the influence of the composition of the medium on the production of EPS by 20 MKB isolates, the following data were obtained: the highest EPS production was obtained in *L. casei* (LCN1) (198.69 mg/l), *L. plantarum* LPN6 (111.85 mg/l), *L. lactis ssp plantarum* LPY8 (196.05 mg/l), *L. coprophilus* COFN1 (185.7 mg/l), *L. brevis* LBN1 (161.35 mg/l) in a medium prepared on the basis of whey (Bukola *et al.,* 2008).

Bergmaier (2005) provide data that EPS containing unusual monosaccharides such as L-rhamnose are a source of new oligosaccharides and valuable substrates for pharmaceutical and aromatic substances (Bergmaier *et al.,* 2016). In this regard, research on increasing the yield of EPS production of the *Lactobacillus plantarum* EB-2 culture is of great scientific and applied importance.

The aim of this work is to establish the optimal cultivation conditions for the biosynthesis of exopolysaccharide by the of *Lactobacillus plantarum* EB-2 strain.

## 2. MATERIALS AND METHODS

The *Lactobacillus plantarum* EB-2 strain was isolated from the Armenian feta cheese, identified by the morphological-biochemical, physiological, as well as 16S rRNK sequencing and deposited in the collection of the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan.

Universal primers of the 16S rRNA gene used in the identification of *Lactobacillus plantarum* EB-2.

| **Primer** | **Primer nucleotide sequence** |
|---|---|
| 16S rRNA2-F | TCG CTA GTA ATC GCG GAT CAG C |
| 16S rRNA2-R | GCA TAT CGG TGT TAG TCC CGT CC |
| 16S rRNA1-F | TCT CAG TTC GGA TTG TAG GC |
| 16S rRNA1-R | ATC GAC TCC TAG TGT CAA GG |

BLAST analysis of the obtained nucleotide sequence of 16S rRNA gene of *L.plantarum* EB-2 was performed using the NCBI database. For the compilation of the phylogenetic tree, 15 strains of *Lactobacillus plantarum* were selected according to the Clustal W program.

The phylogenetic tree was compiled using the Simple Phylogeny online program (www.ebi.ac.uk).

Isolation of the polysaccharide from the culture liquid of *L. plantarum* EB-2 was carried out according to the method described in the work of Cerning (1994). The of *L. plantarum* EB-2 strain was recovered from the freeze-dried state by 2-3 subcultures in MRS broth and incubation at 37$^{o}$C for 48 hours. Inoculum in a volume of 20 ml (2%, w/v) was introduced into 1 liter of fermentation medium. After incubation, TCA was added to the culture liquid to a final concentration of 4% (w/v) and stirred for 30 minutes at room temperature. Cells and precipitated proteins were removed by centrifugation at 7,000 x g for 30 minutes at 4°C. Chilled absolute ethanol was added to the supernatant in an equal volume and kept at 4°C for 48 hours. The precipitated EPS was collected by centrifugation at 7,000 x g for 30 minutes at 4°C. The precipitate was dissolved in distilled water and dialyzed at 4°C for 48 hours and then dried by lyophilization.

To establish the optimal cultivation conditions for maximum EPS production by the strain *L. plantarum* EB-2, studies were carried out to study the effect of various carbon sources (glucose, lactose, sucrose and mannose; 20 g/l), nitrogen sources (tryptone and peptone; 10 g/l), fermentation temperature (30, 35, 37 and 39$^{o}$C), fermentation time (24, 48 and 72 hours) and initial pH value of the medium (5.0; 5.5; 6.0 and 6.5) (Wang *et al.,* 2017). A medium based on curd whey was used as a fermentation medium (Karapetyan *et al.,* 2008). The culture was grown in a shaker-incubator at 100 rpm.

## 3. RESULTS AND DISCUSSION

**Identification of the EPS-producing culture of *L. plantarum* EB-2.** Cells of *L.plantarum* EB-2 culture are Gram-positive, catalase negative short bacilli, located singly and in pairs, 0.6 µm x 1.2-3.6 µm in size. The culture actively ferments salicin, mannose, mannitol, melibiosis, ribose, raffinose, trehalose, sucrose, fructose, lactose and galactose, grows with 6.5% NaCl and 0.4% bile. The *L. plantarum* EB-2 culture was also identified by sequencing and phylogenetic analysis of the 16S rRNA gene. BLAST analysis of the sequence of the 16 S rRNA genes showed high identity (99%) with 15 typical strains of *L. plantarum*. Phylogenetic analysis revealed that the sequencing product from *L.plantarum* EB-2 is very close to the strain *L.plantarum* SRCM103411 (Fig. 1).

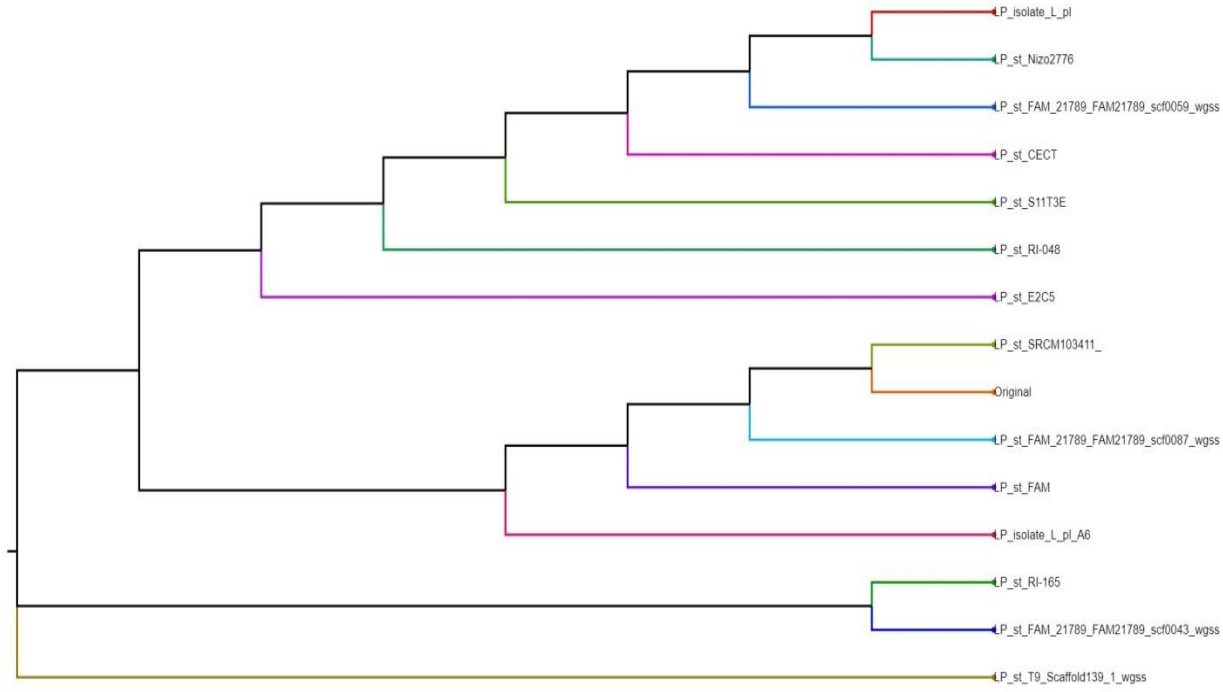


**Fig. 1. Phylogenetic tree, compiled from sequencing of 16 S rRNA genes of *L. plantarum* strains.**

The resulting phylogenetic tree showed that *L. plantarum* strains can be grouped into three separate branches, of which one branch is divided into two branches. Strain *L.plantarum* EB-2 formedas one of the branches alongwith another branch of the strain *L.plantarum* SRCM103411.Within this branch *L.plantarum* EB-2 showed 100% sequence identity with *L. plantarum* FAM 21789 FAM21789 scf0087, *Lactobacillus plantarum* isolate *L. plantarum* A6, *Lactobacillus plantarum* strain Nizo2776 NODE, *L.plantarum* SRCM103411, proving that these five strains are closely related. Sequence identity with the other strains of the cluster was 98%.

**Influence of various nitrogen and carbon sources on EPS production by *L. plantarum* EB-2 strain.** When conducting experiments to study the effect of various nitrogen sources, tryptone and peptone were added to the composition of the fermentation medium. Of the studied nitrogen sources - tryptone and peptone in an amount of 10 g/l - peptone turned out to be the most suitable nitrogen source for the formation of EPS in a medium based on curd whey. When tryptone is used, *L. plantarum* EB-2 culture synthesizes 818.32 mg/l EPS; and when peptone was added to the medium, the production yield was up to 850.61 mg l, which is 32.29 mg/l more than when tryptone was used. When selecting an effective carbon source for EPS formation by the *L. plantarum* EB-2 strain, it was found

that sucrose is the most effective carbon source (Fig. 2).

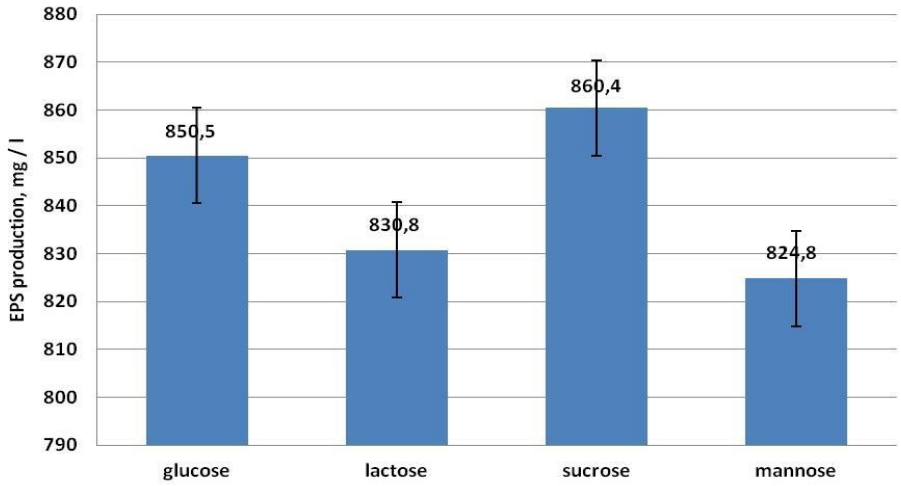


**Fig. 2. Influence of various carbon sources on EPS production *by L. plantarum* EB-2 culture**

In terms of efficiency, carbon sources are located in the following sequence: sucrose>glucose>lactose>mannose. Our results agree with the data of Emanuel Vamanu et al. (2010), who investigated the effect of three types of carbohydrates (glucose, lactose, and sucrose) on EPS production by *L. paracasei* IL2 and *L. plantarum* IL3 strains. His results showed that sucrose is the most suitable carbon source for the synthesis of the EPS of *L. paracasei* IL2. Sucrose and glucose had the same effect on EPS formation by *L. plantarum* IL3 (Vamanu *et al.,* 2010).

According to the results of studies by other authors, glucose and lactose are the most effective carbon sources in comparison with sucrose (Fukuda *et al.,* 2010; Yuksekdag *et al.,* 2008).

Most of the studied *L. plantarum* cultures synthesize from 0.059 to 0.429 g/l EPS, with the exception of some strains that synthesize up to 1 g/l EPS. At the beginning, the amount of EPS of *L. plantarum* KX041 formed by the culture did not exceed 200 mg/l. Thanks to the methods for selecting the optimal values of the cultivation conditions and the components of the fermentation medium, the formation of EPS reached 599.52 mg/l (almost 3 times more) (Wang *et al.,* 2017).

In general, the medium based on cottage cheese whey according to Karapetyan turned out to be the most suitable medium for the formation of EPS by the *L. plantarum* EB-2 strain. It was found that the biosynthetic activity is stimulated by the high content of manganese sulfate in this medium (1.8 g/l). According to the literature, $Mn^{2+}$ regulates cell growth and the activity of enzymes involved in EPS biosynthesis, contributing to an increase in EPS formation (Wang *et al.,* 2017).

**Selection of optimal cultivation conditions for maximum EPS production by *L. plantarum* EB-2 strain.**

The study of the influence of cultivation conditions, such as the time and temperature of fermentation and the initial pH of the medium, showed that these parameters strongly affect the production of EPS by strains of lactobacilli.

When studying the optimal growing temperature for the production of EPS of *L. plantarum* EB-2 strain, the optimal fermentation temperature was 37°C, at which 830.7 mg/l EPS was formed, at 33°C 834.9 mg/l was synthesized, at 35°C 860.4 mg/l and at 39°C 826.5 mg/l EPS (Fig. 3).

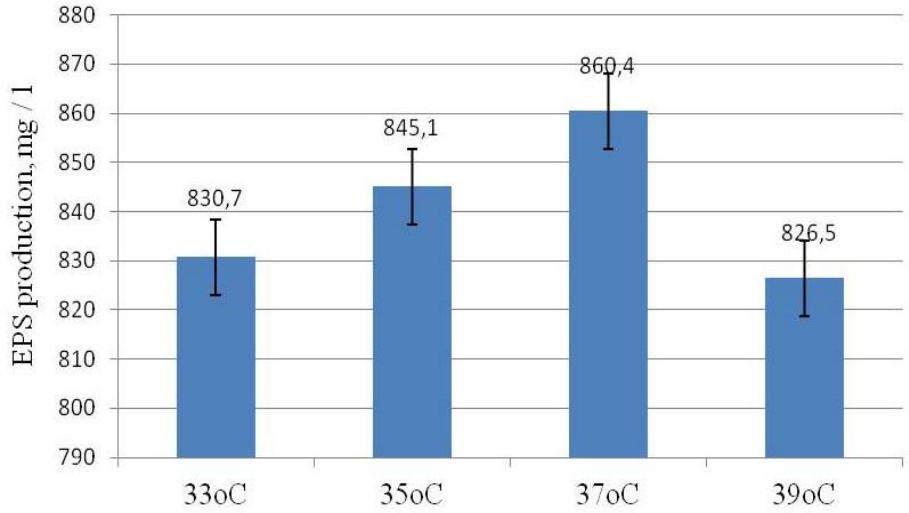


**Fig. 3. Influence of fermentation temperature on EPS production**

**by *L. plantarum* EB-2 strain**

In further experiments, we established the optimal fermentation time. *L. plantarum* EB-2 culture was grown in a medium based on curd whey with pH 6.5 at 37°C for 24, 48 and 72 hours. During fermentation for 24 hours, the culture formed 850.6 mg/l EPS, within 48 hours 901.7 mg/l and within 72 hours 825.6 mg/l EPS (Fig. 4).

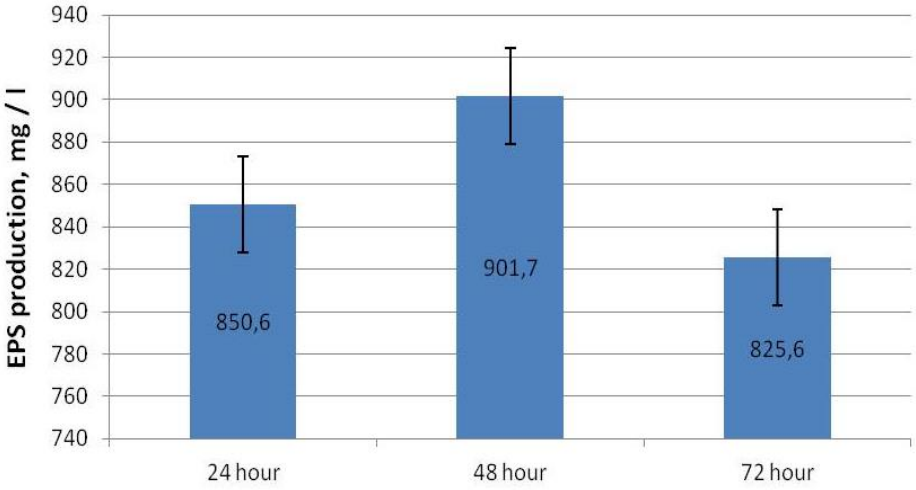


**Fig. 4. Influence of fermentation time on EPS production by the *L. plantarum* EB-2 strain**

After establishing the optimal fermentation time, studies were carried out to determine the optimal values of the initial pH of the medium. The culture of *L. plantarum* EB-2 at 37°C for 48 hours at pH 5.0 formed 890.6 mg/l, at pH 5.5 it formed 910.3 mg/l, at pH 6 it formed 905.5 mg/l and at pH 6.5 901.7 mg/l EPS. Thus, the optimal pH value of the medium is 5.5 (Fig. 5).

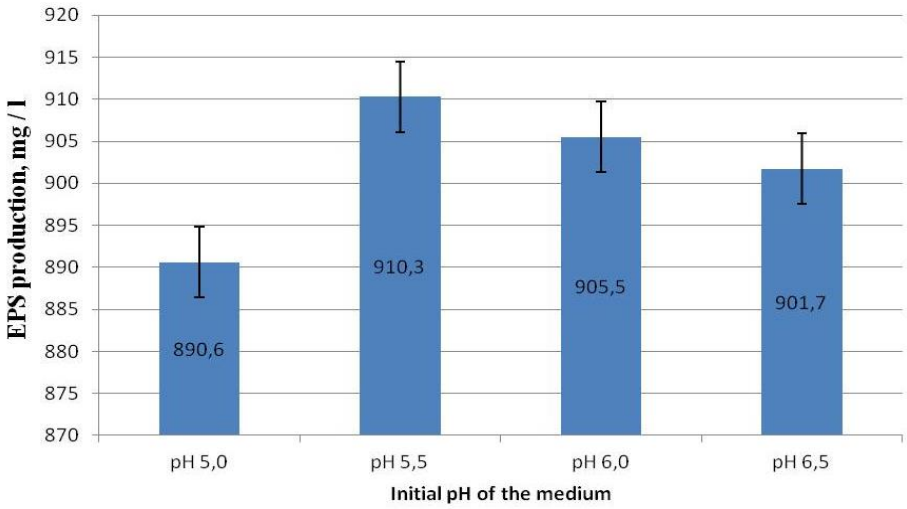


**Fig. 5. Optimal pH values of the medium for the synthesis of EPS by the *L. plantarum* EB-2 strain**

Our data coincide with the data of Khanh (2016). who showed that the synthesis of EPS by the *L. plantarum* T10 strain is the highest when cultivated in an MPC broth with the addition of 4% lactose and 0.4% yeast extract. The above authors showed that the highest EPS yield (417.11 mg/l) was achieved with the following parameters: initial cell density $10^6$ CFU/ml, growth temperature 35°C, pH 5.5, and incubation time 48 h (Khanh *et al.,* 2016).

Thus, we have established that the combination of sucrose and peptone has the greatest stimulating effect on biosynthetic activity. The selected optimal parameters of growing conditions (pH - 5.5; growing temperature - 37°C; fermentation time 48 hours) allow increasing the biosynthesis of EPS by 3-4 times.

## 4. CONCLUSION

As a result of numerous experiments to determine the optimal cultivation conditions, nitrogen and carbon sources for the synthesis of EPS by the *L. plantarum* EB-2 strain, it was found that the cultivation of the culture under the conditions of an incubator shaker, in a medium based on curd whey with a pH of 5.5, at a fermentation temperature 37°C, with a fermentation time of 48 hours, when using peptone and sucrose, we increased the EPS yield to 910.3 mg/l, which is 3.6 times more than the initial volume of formation of EPS without selecting fermentation parameters (250.0 mg/l).

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**Authors' contributions**

All the authors substantially contributed to the conception, compilation of data, checking and approving the final version of the manuscript, and agree to be accountable for its contents.

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There exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

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