Isolation and selection of sauerkraut lactic acid bacteria producing exopolysaccharides

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Abstract. Fermented plant-based foods, including sauerkraut, offer high nutritional and functional value. Their microflora is dominated by lactic acid bacteria which are a source of different substances with health-promoting benefits and diverse applications in the food industry. Production of exopolysaccharides (EPSs) by lactic acid bacteria attracts particular interest in the food industry due to their rheological properties. In the present study, we isolated 20 strains of lactic acid bacteria from traditional Bulgarian sauerkraut. The isolates were identified by 16S rDNA sequencing and were attributed to *Lactiplantibacillus plantarum* (75%) and *Pediococcus pentosaceus* (25%). All strains were screened for their ability to synthesize exopolysaccharides, and 6 of them proved positive. Since culture media composition and especially the carbon source is a critical factor influencing the yield of bacterial EPSs, the impact of various carbon sources on the EPSs synthesis by the selected producers was explored. The best results were obtained by using glucose and sucrose as sole carbon sources.

1 Introduction

Plant-based fermentations foods and their microbe communities provide an underexplored source for novel biotechnological applications [1]. Fermentation is considered one of the oldest methods for food preservation. The microbiota initially present in the processes of lactic fermentation comes mainly from the raw materials, although other ingredients used and the industry's own environment influence its composition [2].

Lactic acid bacteria (LAB) are the key microorganisms in the fermentation of vegetables. Most LAB, especially *Lacticaseibacillus, Lactiplantibacillus, Lactobacillus, Lactococcus, Latilactobacillus, Lentilactobacillus, Leuconostoc, Limosilactobacillus, Pediococcus*, *Streptococcus*, and *Weissella* species are capable of synthesizing a variety of exopolysaccharides (EPS) [3, 4, 5]. They play critical role in stress resistance at single cell level [6]. Exopolysaccharides are long-chain extracellular polysaccharides that are excreted into the extracellular space or remain bound to the bacterial cell surface. They have positive contribution to textural properties. In the food industry, they are used as thickeners, stabilizers, and gelling agents [5]. EPS produced by *Lactobacillus* bacteria also have a number of documented pro-health properties, including anti-cancer, antioxidant, prebiotic, immune-modulating, cholesterol-lowering and other effects [7, 8, 9]. Four hundred different EPS variants with various chemical structures have been published, of which some can be linked to specific strains [10]. Exopolysaccharides from LAB are highly diverse and can be classified following different criteria. The most common criterion is their monomer composition, which allows classifying them into two major groups: homopolysaccharides (HoPS) - composed of one type of monosaccharides, and heteropolysaccharides (HePS) - composed of multiple kinds of monosaccharides [11]. Traditional fermented foods may be a rich source of HoPS-producing LAB [12].

The majority of EPSs produced by LAB are HePS, containing three to eight repeating units composed of two more monosaccharides [4]. It is known that the main determinants of EPS synthesis are culture conditions: growth medium composition, temperature, pH, duration, and so on [13, 14]. However, the first and foremost factor is growth medium composition as it directly affects EPS yield and chemical composition. LAB can produce EPSs with widely varying structures, depending on the strain and on the carbon source in the medium. [13]. On the other hand,

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the optimal composition of the nutrient medium and the choice of a suitable carbon source also have their economic aspect. The aim of the present study was isolation and molecular identification of lactic acid bacteria from sauerkraut and selection of sauerkraut lactic acid bacteria producing exopolysaccharides, as well as selection of an appropriate carbon source for this synthesis.

2 Materials and methods

2.1 Sauerkraut sample

Homemade sauerkraut was used in the present study. White cabbage in mature state was used for its preparation. The obtained cabbage was cleaned of the core and outer leaves. Subsequently, the cabbage was placed in a container with volume 60 L and NaCl was added in the amount 30 g/kg. The closed container was incubated for fermentation at 15 - 20°C for approximately 40 d.

2.2 Isolation, enumeration and phenotypic characterization of LAB

Enumeration and isolation of LAB was performed according to ISO 15214:1998[6]. Dilutions of 10^{-4},10^{-5} and 10^{-6} were used for spread plate inoculations on MRS agar for total LAB and on M17 agar for the isolation and enumeration of cocci. The plates were incubated at 37°C for 48 h. Samples were analyzed in duplicate. From each medium, a number of colonies equal to the square root of the total number recorded in Petri dishes with 15 to 300 CFUs were randomly selected for isolation. The isolated LAB were subjected to phenotypic and genotypic characterization [15]. The phenotypic characterization of LAB was performed based on cell morphology, Gram reaction and catalase activity. Gram staining was performed for all isolated colonies according to the standard procedure [16]. For the catalase test, a drop of 3% hydrogen peroxide was added to a bacterial colony on a sterile glass slide and mixed well. Production of air bubble indicated catalase positive and no bubble indicated catalase negative activity. Only Gram positive and catalase-negative strains were selected for further characterization. Pure cultures were obtained from the samples and stored at -20°C in Microbank™ vials.

2.3 Molecular identification of LAB

2.3.1 Extraction of DNA

The total genomic DNA of the LAB strains were extracted from overnight cultures grown in MRS. DNA extraction was done by Bacterial Genomic DNA Isolation Kit (Canvax Biotech, S.L., Spain) according to manufacturer's instructions. The quality and concentration of DNA extracts were assessed by determination of absorbance at 260 nm and 280 nm (Shimadzu UV-VIS, Shimadzu Corporation, Japan)

2.3.2 Molecular identification of LAB by 16S rRNA sequence analysis.

LAB identification was performed by PCR amplification of the 16S rRNA gene and sequencing of the PCR products. A set of primers were used for PCR amplifications: LacbF – forward primer (5'-TGC CTA ATA CAT GCA AGT – 3') and LacbR – reverse primer (5'-CTT GTT ACG ACT TCA CCC-3'). PCR analysis was performed in final reaction volumes of 20 µl containing 8 µl of Horse-Power™ Red-Taq DNA Polymerase Master Mix (Canvax Biotech, S.L., Spain), 8 µl ddH_2O, 2 µl of DNA, 1 µl of each primer (3 µM). The PCR conditions were: 5 min at 94°C for initial denaturation, followed by 35 cycles of 1 min at 94°C for denaturation, annealing for 45 s at 57°C, primer extension for 2 min at 72°C, and final extension for 5 min at 72°C, performed by a PCR cycler of Applied Biosystems Inc. The obtained amplicons were stained with Safe View (NBS Biologicals, UK) and separated on 1.2% agarose gel for 50 min at 100V using VWR Mini electrophoresis system. The PCR products of approximately 1450 bp were cut out from the gel and purified with Clean-Easy™ Agarose Purification Kit (Canvax Biotech, S.L., Spain). Sequencing of the PCR products was performed by Microsynth Seqlab, Göttingen, Germany. The sequences were analyzed by BLAST software (www.ncbi.nlm.nih.gov/BLAST/) and EZTAXON (www.ezbiocloud.net), and compared with the nucleotide sequences in the gene bank database (www.ncbi.nlm.nih.gov) [17].

2.3.3 LAB screening for exopolysaccharide-producing capacity

The exopolysaccharide-producing capacity of the strains was tested by 24 h cultivation in MRS medium. First, the strains were activated by transferring 1-2 beads from the Microbank™ vials into fresh MRS broth medium and activated at 37°C for 24 h. After activation, test tubes with 10 ml sterile MRS broth were inoculated with 1 ml of the activated cultures and cultivated for 24 h at 37°C. Parallel examination was done at 25°C for 72 h in culture medium for synthesis of exopolysaccharides (Table 1). After that, in the both experiments, the bacterial cells were removed by centrifugation at 4000 rpm for 30 min. Two volumes of chilled 96% ethanol were then added to the supernatant to precipitate the EPSs. All samples with precipitate in the tubes were determined as positive.

2.3.4 A comparative study of the influence of carbon source on exopolysaccharide synthesis

All positive strains in MRS broth were tested in culture medium for effective synthesis of exopolysaccharides with 3 different carbon sources (sucrose, glucose and fructose). The concentration of each saccharide in the medium was 20 g/L. The components of the culture medium for synthesis of exopolysaccharides are presented in Table 1.

Table 1. Culture medium for synthesis of exopolysaccharides [18]

Component	g/L
K_2HPO_4	2.0
Sodium acetate	5.0
Tri–ammonium citrate	2.0
$MgSO_4$	0.2
$MnSO_4$	0.05
Carbon source (fructose, glucose or sucrose)	20.0
Yeast extract	4.0
Tween 80	1.0
Initial pH	5.7

The same test was also carried out with MRS broth. Test tubes with 10 ml sterile culture medium were inoculated with 1 ml of activated cultures (1.5 - 7.3 × 10^8 cfu/ml) and cultivated for 72 h at 25°C. After cultivation, bacterial cells were removed by centrifugation at 4000 rpm for 30 min. Two volumes of chilled 96% ethanol: isopropanol mix (1:1) were then added to the supernatant to precipitate the EPSs.

The alcoholic supernatant was homogenized very well and the samples were incubated at 4°C for 24 h. After incubation, polysaccharides were separated by filtration and then filters were dried at 37°C for 48 h to a constant weight and the dry weight was expressed in gram per millilitre. Each sample was analyzed in triplicate.

3. Results and discussion

3.1 LAB isolation, enumeration and characterization

The reference method ISO 15214:1998 was used to isolate LAB from sauerkraut [19].

Table 2. Phenotypic characteristics of the colonies

Isolate	Colony morphology	Gram-reaction	Catalase activity
ZJ1, ZJ2, ZJ3, ZJ4, ZJ5	Small, white, circular and convex colonies with smooth margins and viscid consistency.	G (+)	C (-)
ZM1, ZM2, ZM3, ZM4, ZM5, ZM6, ZM7, ZM8	Small, yellow, circular and convex colonies with undulated margins and dry consistency.	G (+)	C (-)
ZS1, ZS3, ZS4, ZS5, ZA3, ZH2, ZE2	Small, white, circular and convex colonies with smooth margins and viscid consistency.	G (+)	C (-)

A total of 20 isolates were obtained from plating on MRS agar and M17 agar. MRS agar counts were 9.2 × 10^7 cfu/g and M17 agar harboured 1.5 × 10^8 cfu/g. The predominant colonies on MRS agar were yellow, small and smooth, circular in form with undulate margins and convex elevation. When manipulated with a needle, the colonies were viscous. On the other hand, the predominant colonies from M17 agar were small, white and convex, with smooth margins. They also had viscid texture. The isolates were phenotypically characterized on the basis of their cell morphology, Gram reaction and catalase activity. All isolates were Gram-positive and catalase negative. Microscopic observation showed that all bacteria from ZM-series and ZS-series were rods, and bacteria from the ZJ-series were cocci.

3.2 Molecular identification of LAB by 16S rRNA sequence analysis

Identification of the phenotypically characterized strains was performed by PCR amplification of the 16S rRNA gene and sequencing of the PCR products.

Table 3. Molecular identification of lactic acid bacteria from sauerkraut

LAB isolates	ID	Level of similarity, %
ZJ1	*Pediococcus pentosaceus*	99.66
ZJ2	*Pediococcus pentosaceus*	99.58
ZJ3	*Pediococcus pentosaceus*	99.66
ZJ4	*Pediococcus pentosaceus*	99.28
ZJ5	*Pediococcus pentosaceus*	99.74
ZM1	*Lactiplantibacillus plantarum*	97.39
ZM2	*Lactiplantibacillus plantarum*	99.57
ZM3	*Lactiplantibacillus plantarum*	99.83
ZM4	*Lactiplantibacillus plantarum*	99.56
ZM5	*Lactiplantibacillus plantarum*	100.00
ZM6	*Lactiplantibacillus plantarum*	99.83
ZM7	*Lactiplantibacillus plantarum*	99.40
ZM8	*Lactiplantibacillus plantarum*	99.40
ZS1	*Lactiplantibacillus plantarum*	98.40
ZS3	*Lactiplantibacillus plantarum*	99.66
ZS4	*Lactiplantibacillus plantarum*	99.33
ZS5	*Lactiplantibacillus plantarum*	99.66
ZA3	*Lactiplantibacillus plantarum*	99.74
ZH2	*Lactiplantibacillus plantarum*	99.24
ZE2	*Lactiplantibacillus plantarum*	99.77

The resulting sequences were processed and after BLAST analysis the species identity was determined. All isolates from ZJ-series were attributed to *Pediococcus pentosaceus*, while the rods from ZS-series and ZM-series were *Lactiplantibacillus plantarum.* High levels of similarity were obtained both for the *Pediococcus pentosaceus* strains (99.28% - 99.74%) and *Lactiplantibacillus plantarum* (97.39% - 100.00%).

The most commonly found lactic acid bacteria species in sauerkraut *are Lp. plantarum, L. pentosus, Lc. mesenteroides, L. brevis, L. sakei, L. curvatus, L. paraplantarum, L. coryniformis, P. pentosaceus, Lc. citreum, Lc. argentinum,* and *Weissella* sp. [2]. *Lactiplantibacillus plantarum* is the most frequently isolated LAB species from fermented vegetables [20].

Our results confirm the predominant presence of *Lp. plantarum* and *Pediococcus pentosaceus* as the main LAB species in the studied sauerkraut. In spontaneous sauerkraut fermentation, *Leuconostoc mesenteroides* initiate the fermentation process, followed by the growth of other lactic acid bacteria (LAB), mainly *Lactobacillus brevis*, *Pediococcus pentosaceus*, and *Lactiplantibacillus plantarum*, where *Lp. plantarum* is responsible for the second phase of fermentation and the high acidity of the produced sauerkraut [21].

3.3 LAB screening for exopolysaccharide-producing capacity

EPSs produced by *Lp. plantarum* in the fermented products have received considerable attention due to their biological activity and health benefits [22].

Exopolysaccharide producers can be identified by their phenotypes on solid and in liquid media [10]. In our study, working with a liquid culture medium was preferred. Further, all 20 isolates were screened for the production of EPSs in liquid medium. The screening was first performed in tubes containing liquid medium MRS broth for 24 h at 37°C. Subsequent research showed that the medium described in Table 1 is much more suitable for the screening experiment. The best EPS-producing capacity of lactic acid bacteria has been observed in media with limited nutrient composition. On the other hands, temperature is a critical factor in the synthesis of polysaccharides. The best results for EPS production were obtained at 25°C. After the initial screening in MRS broth, a second screening experiment was performed in the medium with limited composition (table 1) with strain cultivation at 25°C for 72 h. The production of polymers in both experiments was confirmed by mixing the supernatant of each culture with alcohol. Precipitate formation indicated the presence of EPS. In this screening, six LAB strains (ZS1, ZS3, ZS4, ZS5, ZH2, ZE2) demonstrated ability to produce EPS in different amounts. All of them were identified as *Lactiplantibacillus plantarum.*

3.4 A comparative study of the influence of carbon source on exopolysaccharide synthesis

The composition of nutrients is one of the factors which affects the growth and metabolism of cells [23].

Optimization of the growth medium is critical to achieve maximum EPSs productions by LAB [4]. Sucrose and glucose are commonly used for the isolation of EPS-producing bacteria; they provide the greatest production of biopolymers in most bacteria [24]., The relatively similar results for glucose and sucrose were achieved of the three carbon sources tested.

The used carbon source affects the composition of the synthesized exopolysaccharide. In our study, the six positive *Lp. plantarum* strains were tested for their ability to synthesize EPSs in the medium described in table 1, with the use of three different compounds as single carbon sources - glucose, fructose and sucrose. MRS broth was used as a control medium. For the majority of the strains, no significant difference was observed in the amount of exopolysaccharides produced when sucrose and glucose were used as single carbon sources. Strain ZE2 was an exception, with higher levels of EPSs obtained with sucrose as a carbon source. The levels of exopolysaccharides obtained with fructose were much lower. These results prove that fructose is unsuitable as a carbon source for the studied strains.

Fig. 1 shows significant differences in the amount of the produced exopolysaccharides between individual strains under the same carbon source levels. Compared to control medium, results obtained with the medium for synthesis of exopolysaccharides (with glucose and sucrose) show lower levels of EPSs, with the exception of strains ZE2 and ZH2.

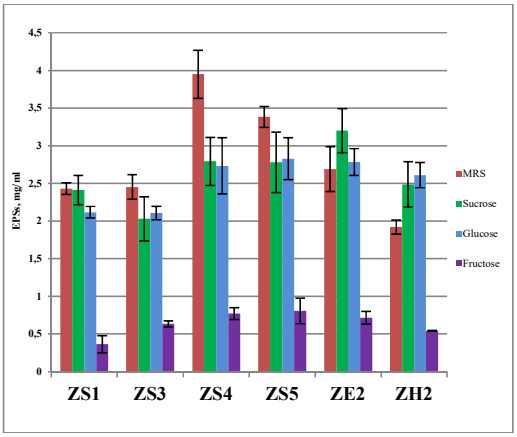

Fig.1. Influence of carbon source on exopolysaccharide synthesis

Comparing the control MRS broth and the exopolysaccharide synthesis medium with sucrose, differences in the amount of exopolysaccharide were: 5.12% (ZS1), 17.25% (ZS3), 29.28% (ZS4) and 17.83% (ZS5) in favour of the control medium. Similar levels of exopolysaccharide synthesis were observed with glucose as a carbon source. The content of exopolysaccharides was 16,77% (ZS1), 14.13% (ZS3), 30.8% (ZS4), and 16.35% (ZS5) higher in the control medium compared to the medium for synthesis with glucose.

Although higher results were obtained with the control medium for some of the strains, the isolation and purification of the resulting exopolysaccharide was

challenging. It was much faster and more efficient to isolate exopolysaccharides from the exopolysaccharide synthesis medium (Table 1). In addition, the exopolysaccharide synthesis medium is much cheaper. The situation is different with ZE2 and ZH2, where the amount of the exopolysaccharides was 18.96% (ZE2) and 29.52% (ZH2) higher than the exopolysaccharides from the control medium where the carbon source is sucrose.

The amount of exopolysaccharides produced with these two sugars is much higher compared to the use of fructose. LAB are able to utilize sucrose and glucose more efficiently compared to other sugars as an energy source or precursor for the EPS synthesis [25]. Zotta et al. [26] reported that sucrose was one of the most suitable carbon sources for EPS production by LAB. That is why we chose for the present study a medium with a limited nutrient composition with glucose or sucrose as the main carbon source. A trial with fructose was also performed. This medium is more than four times cheaper than the standard MRS medium and is easy to prepare [13]. Higher LAB growth and greater production of polysaccharides occurred in the range of 25°C to 35°C [24]. The initial screening was performed at 37ºC, and subsequently at 25ºC, but with a longer duration. The amount of the synthesized exopolysaccharide at 25ºC was visibly greater. Therefore, 25ºC was chosen as the temperature at which to perform the comparative study. Temperature is a factor influencing the amount of exopolysaccharide production, while the composition of the medium determines EPS composition and structure. Precipitation with alcohols or acetone is a common EPS purification and isolation approach [27]. The efficiency of polymers precipitation depends on their chemical structure, molecular weight, and the final concentration of polymer and alcohol used for precipitation [28]. Polysaccharides usually precipitate as fibers when alcohols such as ethanol or 2-propanol are used as precipitant [10]. In addition to the choice of alcohol for precipitation, its quantity and very good homogenization of the mixture are also important factors. Most EPSs are highly soluble in aqueous solutions, whereas the solubility can be drastically decreased by using water miscible solvents by extracting water molecules from the hydration shell [10]. The use of two or more volumes of alcohol and the more intensive homogenization results in more effective precipitation. The precipitation process is fast, easy to handle and at the same time purifies the EPS from the remaining sugars and salts of the cultivation media [29]. EPS production is strain-specific [24], which may explain the ability of ZS-series to produce EPSs, in contrast to the ZM-series strains, despite that the all strains in the two LAB series belong to the same species (*Lp. plantarum)*.

4 Conclusions

The lactic acid bacteria isolated from sauerkraut on our study are of the most common species usually associated with sauerkraut. Our results confirmed that *Lp. plantarum* and *P. pentosaceus* are typical for the end of the fermentation process in sauerkraut, with *Lp. plantarum* observed as the predominant species. Although exopolysaccharide producers are found among both LAB species, the ability for EPS production was confirmed to be strain-specific. Culture media composition and especially the carbon source are critical factors influencing the yield of bacterial EPSs. Using glucose and sucrose as sole carbon sources in a medium with limited nutrient composition allows easy and inexpensive screening for EPS synthesis and the isolation of exopolysaccharides from the lactic acid bacteria culture broth.

The research presented in this paper was supported by KP – 06 – COST/11 - 29.07.2022

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