Biotechnological studies on the production of exopolysaccharides by lactic bacteria in batch system

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Abstract

The purpose of the research was to highlight and determination of the exopolysaccharides quantity produced by 2 lactic bacteria strains (Lactobacillus plantarum 2s and Streptococcus thermophilus 1t). The biosynthesis process was performed in a bioreactor, on media containing 5% and 10% glucose, at 40^{o}C. The exopolysaccharides production and the glucose consumption were determined and the biomass grows was graphically represented on the three culture media. On the basis of biochemical methods, we determined the glucose and galactose content of the exopolysaccharides produced by the 2 strains.

Keywords: *Lactobacillus plantarum* 2s, *Streptococcus thermophilus* 1t, exopolysaccharides, glucose, corn extract

Introduction

Most of the microorganisms synthesize several types of polysaccharides, which differ depending on their localization in the cell. The polysaccharides that are localized inside the cell, at the cytoplasm level, are used by the producing strains as an energy source. Other polysaccharides are localized outside the cell, having a protecting role. The lactic bacteria strains are important producers of exopolysaccharides. These bacteria are important, from the viewpoint of the exopolysaccharides biosynthesis, due to the pharmaceutical implications and to their use in the manufacture of fermented foods containing only natural ingredients. [1]

The lactic bacteria that produce exopolysaccharides isolated themselves from various ecosystems. However, the production largely depends on the composition of the culture media or on the variations of the environment they are in. This is very important because it influences the role these exopolysaccharides are going to have. [2] Generally, lactic bacteria are not capable of catabolizing the produced exopolysaccharides, as these latter do not have an energetic role for the microbial cell. One of the main roles of the exopolysaccharides produced by lactic bacteria strains is to favor the adherence of the microbial cell to the intestinal cells, favoring its colonization. [3]

The microbial origin exopolysaccharides have always been in competition with the synthetically obtained ones. Even if the role of microbial exopolysaccharides is more important, the chemical synthesis products are more accessible due to the manufacture simplicity [4]. The most important traded exopolysaccharide is the xanthan, which is successfully used as a food additive, being synthesized by a bacterium (*Xanthomonas campestris*). The exopolysaccharides produced by lactic bacteria are very important also due to the role they play in human health [5]. Due to their use in the manufacture of fermented milk products, lactic bacteria are an important group in alimentation. These lactic bacteria confer important properties to products. Another important factor is the capacity to biosynthesize lactic acid; therefore, these products do not need the presence of certain chemically obtained food preservatives, very harmful to the human body [6].

Most of the research was made on the media containing milk, because the production of exopolysaccharides significantly influences the final product. Many references are made to the *Lactobacillus delbrueckii* or *Lactobacillus bulgaricus* strain as a good producer under the influence of the casein in milk, added to the culture media. [7] The same production is noticed in *Streptococcus thermophilus* strains, if they are cultivated on milk. In the case of other lactic bacteria strains, the yeast extract or various amino-acids are

mentioned as nitrogen sources with positive influence on the production of exopolysaccharides. In this situation, the corn extract may form a viable source of nitrogen [8].

The carbon source, by its quantity and combinations, significantly influences the production of polysaccharides. Most of the research work indicates glucose as the most significant influence carbon source. [9] Many research works are mentioned that prove that other monosaccharides also have the same effect in the case of *Streptococcus thermophilus* strains. In exchange, it can be noticed that lactose or galactose does not influence the results significantly. Thus, we can conclude that the production of exopolysaccharides depends very much on the used strain and, then, on the carbon source [10, 11].

For most lactic bacteria, the composition of the culture medium and the influence of the cultivation temperature and of the pH are determining factors in the exopolysaccharides synthesis. To conclude, biosynthesis depends on the bacterial strain, on the composition of the medium, and on the cultivation conditions. Another important aspect is the moment when the synthesis takes place. Usually, in happens during the logarithmic increase stage and during the stationary stage. The maximum synthesis time is specific to the used microorganism. It can take place during any of the two increase stages or during both increase stages. Thus, the production of exopolysaccharides is maximal under the particular conditions of each lactic bacterium strain. [12]

Materials and Methods

Microorganisms. To perform the studies, 2 lactic bacteria strains were used: *Lactobacillus plantarum* 2s and *Streptococcus thermophilus* 1t, kept in the collection of the Biotechnology Faculty, USAMV Bucharest and of the Biotehnol Center of Bucharest. The strains are kept at -82^{o}C, in specific MRS and LIA media, supplemented by 20% glycerol. To obtain the pre-inoculum, a volume of 20 ml of MRS and LIA is sowed with 0.1 ml of culture kept in the freezer. The sowed volumes are kept for 24 hours at 40^{o}C. The inoculum is prepared by sowing a second volume of 20 ml of MRS and LIA with 1%, both of them being kept for 12 hours at 40^{o}C. The resulting cultures are used in obtaining larger volumes of microbial inoculum for the tests at the laboratory level and at the Biotech-Braun bioreactor level, 3 liters of efficient volume.

Culture media. To perform the studies, 3 formulas of culture media were used. These culture media contain (g/v): 5% and 10% glucose, 1% corn extract (40% dry matter). The culture media were noted PGD5, PGD10, depending on the quantity of glucose they contain. The pH of the media was established at 5 – 5.5 and the sterilization was performed at 115^{o}C for 20 minutes.

Fermentation in the Biotech-Braun bioreactor. The fermentations were performed in Biotech-Braun bioreactor with a working capacity of 3 liters of medium. During the fermentation, the pH was maintained around the value of 5 with NaOH 20%. After 12 hours from cultivation, a stirring of 150 rpm was performed for 30 minutes. The cultivations length was of 72 hours. Samples were collected from the bioreactor every 4 hours, in volumes of 15 ml. The biomass grows, the glucose content and the production of exopolysaccharides were determined.

Determination of the biomass grows. It was performed spectrophotometrically, at 600nm, with a Hiro spectrophotometer. Before reading the results, a dilution of 1:10 is made.

Determination of the glucose quantity by using the o-toluidine method. It was performed by the o-toluidine test, made by the National Institute of Chemical-Pharmaceutical Research-Development – ICCF Bucharest.

Determination of the galactose quantity. The galactose is indirectly dosed by measuring the quantity of $NADH+H^{+}$ in the reaction, because the obtained quantity of $NADH+H^{+}$ is equivalent to the quantity of galactose in the sample. [13]

Determination of the lactose quantity. The lactose is hydrolyzed to D-glucose and D-galactose at pH 6.6, in the presence of β-galactosidase. [13]

Determination of the lactic acid quantity. The accumulation of lactic acid was determined by titration with HCl 0.1N. For the determination, the fact that 1 ml NaOH 0.1N corresponds to 0.009008 g lactic acid is taken into account.

Determination of the exopolysaccharides quantity and their partial purification. The cells are removed from the samples by centrifugation at 11,000 rpm, for 10 minutes. To the supernatant are added 2

volumes of ethanol kept in the freezer, and the mixture is kept overnight at 4^{o}C. The precipitate is isolated by centrifugation at 3000 rpm, for 15 minutes, then washed once more in cold ethanol. The total sugar content of exopolysaccharides was determined by using the phenol-sulfuric acid method. [14]

Results and Discussions

For the inoculation of the bioreactor with 1% culture of *Lactobacillus plantarum* 2s and *Streptococcus thermophilus* 1t, an appropriate inoculum volume was prepared, obtained by using the MRS and LIA media, respectively. In the case of this inoculum, the biomass grows, the lactic acid accumulation and the sugar consumption specific to each type of culture medium were determined.

Figure 1 shows that the lag stage is extremely short (maximum 4 ore), which is due to the use of the optimal inoculum that leads to the decrease of the glucose level and to the synthesis of the lactic acid. The *Lactobacillus plantarum* 2s strain has a logarithmic increase stage of up to 20 hours of fermentation, when it gradually passes into the stationary stage. This slowing down of the increase rhythm is correlated with the decrease of the lactic acid synthesis and with the more and more low consumption of glucose. Even if the glucose continues to decrease even after 16 hours, the synthesis of the lactic acid is stopped for the main part, the glucose consumption being able to be associated to the support of the number of microorganisms in a medium with a smaller and smaller pH.

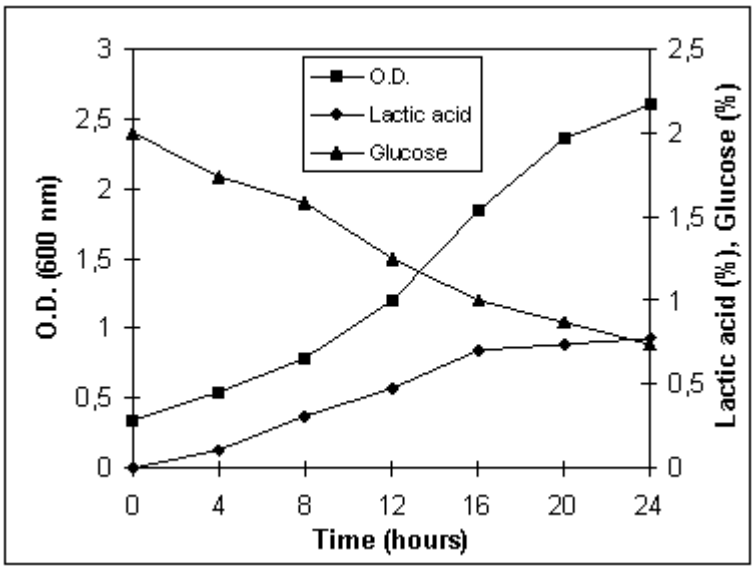


Figure 1. Biomass grows, lactic acid accumulation and glucose consumption for the *Lactobacillus plantarum* 2s strain

For the *Streptococcus thermophilus* 1t strain (**Figure 2**), the logarithmic increase stage lasts also up to 20 hours of fermentation. The maximum increase speed is somewhere within the interval of 8 – 20 hours of fermentation. The synthesis of the lactic acid lasts until up to 16 hours of fermentation. In general, within 24 hours, the lactose consumption is constant, consuming only 50% of the inserted quantity. After approximately 24 hours of fermentation, the strain passes into the stationary stage, wherefrom it follows that a permanent control of the pH, even in the case of an inoculum, leads to unwanted results.

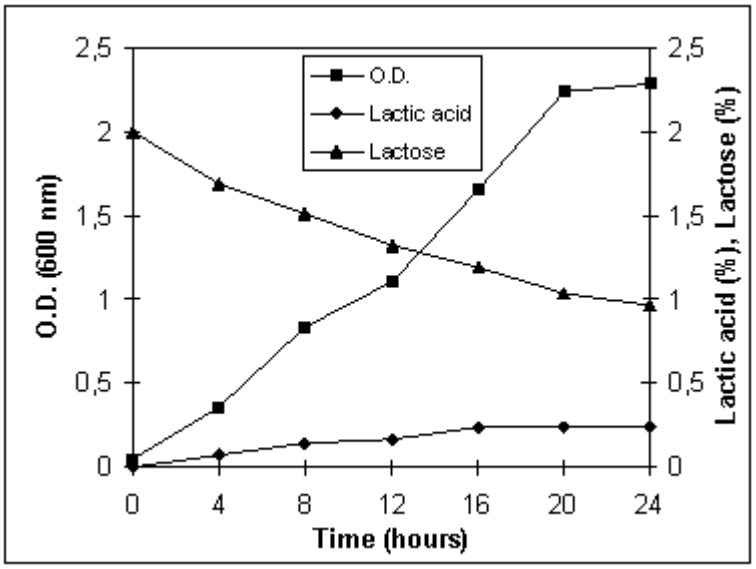
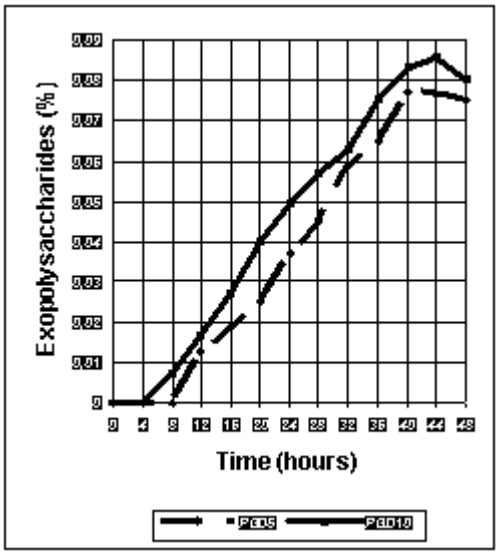


Figure 2. Biomass grows, lactic acid accumulation and lactose consumption for the *Streptococcus thermophilus* 1t strain

For the cultivation in the bioreactor, a fresh 24-hour inoculum is used. During fermentations, the pH was permanently corrected with NaOH 20%, in order not to negatively influence the production of exopolysaccharides of the two strains. **Figure 3** shows the results of the biosynthesis of exopolysaccharides, the glucose consumption and the biomass grows on the 2 media (PGD5, PGD10) with the *Lactobacillus plantarum* 2s strain.



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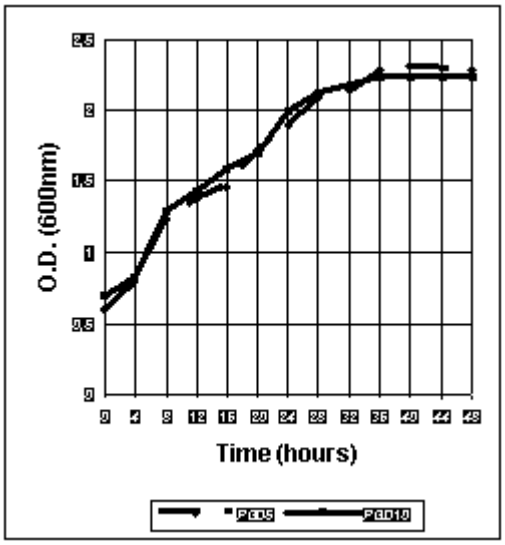

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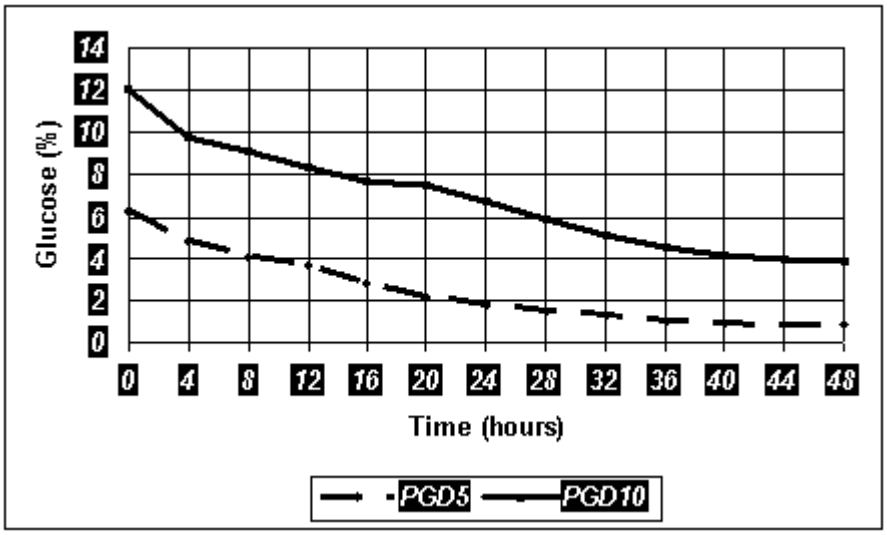

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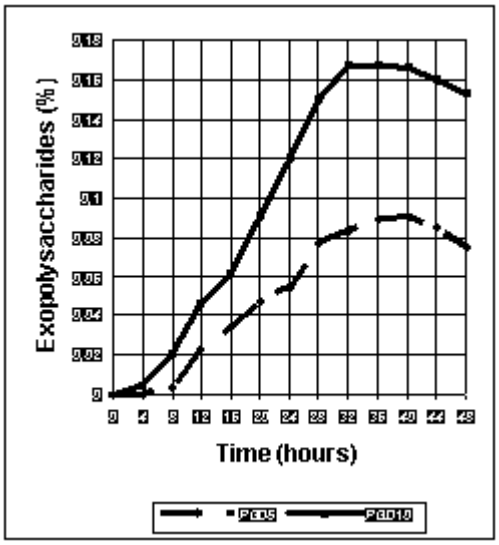
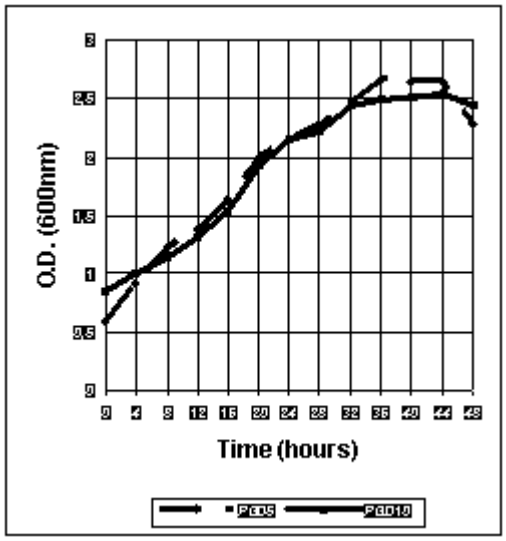
Figure 3. Results of the synthesis of exopolysaccharides (A.), of the biomass grows (B.) and of the glucose consumption (C.) for the *Lactobacillus plantarum* 2s strain when cultivated in a bioreactor

By analyzing the 3 graphs it results that better results were obtained when using the PGD10 medium. Using a double glucose quantity (10%) determines the decrease of the logarithmic increase period by 2 – 4 hours (**Figure 4B**). The quick formation, in a significant quantity, of the lactic acid inhibits the strain evolution more quickly. The synthesis of exopolysaccharides on the PGD10 medium continues until the middle of the stationary stage (**Figure 4A**). The glucose consumption is constant, and less than half of the inserted glucose remains after 48 hours of fermentation (**Figure 4C**).



A.



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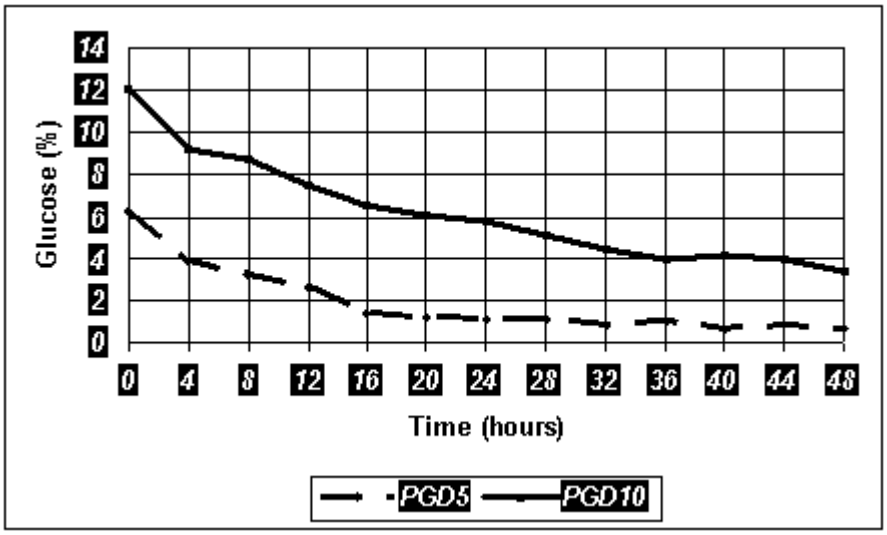

C.

Figure 4. Results of the synthesis of exopolysaccharides (A.), of the biomass grows (B.) and of the glucose consumption (C.) for the *Streptococcus thermophilus* 1t strain when cultivated in the bioreactor

Compared to the *Lactobacillus plantarum* 2s strain, the production of exopolysaccharides is larger for the *Streptococcus thermophilus* 1t strain, particularly when the PGD10 medium is used (**Figure 4A**). The lag stage is extremely short, compared to the *Lactobacillus* strain. The *Streptococcus thermophilus* 1t strain passes into the stationary stage 36 hours after fermentation, in the case of both medium formulas. The glucose consumption is lower, which is noticed during the first 20 hours of fermentation. This period corresponds to the synthesis accentuated by the lactic acid and by exopolysaccharides (**Figure 4B** and **4C**).

The evolution of the kinetic parameters during the fermentations obtained in the Biotech-Braun bioreactor is shown in **Table 1**. The main conclusion of the results obtained is the very good productiveness of the *Streptococcus thermophilus* 1t strain, compared to that of the *Lactobacillus plantarum* 2s strain. By using the same composition of the culture medium in the bioreactor, the productiveness of the *Streptococcus thermophilus* 1t strain is at least 50% better than that of the *Lactobacillus plantarum* 2s strain. Even if *Streptococcus thermophilus* 1t is known as an important producer of exopolysaccharides, the lack o the yeast extract or of other similar compounds has a great importance on the *Lactobacillus plantarum* 2s strain. If, by using the PGD5 medium, the quantity of exopolysaccharides obtained is not very different from the *Streptococcus thermophilus* 1t strain, in the case of the PGD10 medium, the *Lactobacillus plantarum* 2s strain produced approximately half of the quantity produced by *Streptococcus thermophilus* 1t.

Table 1. Kinetic parameters of exopolysaccharides with a batch culture of *Lactobacillus plantarum* 2s and *Streptococcus thermophilus* 1t

Strain	Culture medium	Initial glucose (%)	Quantity of exopolysaccharides (%)	μ_{max} (h^{-1})	Productiveness (g/L×h)
Lactobacillus plantarum 2s	PGD5	6,2	0,077	0,0325	0,78
	PGD10	12	0,0855	0,02875	0,69
Streptococcus thermophilus 1t	PGD5	6,2	0,09	0,0725	1,16
	PGD10	12	0,1672	0,085	2,04

Table 2. Composition of the exopolysaccharide monomers produced by *Lactobacillus plantarum* 2s and *Streptococcus thermophilus* 1t

Strain	Composition of the exopolysaccharide monomers (mg/l)
Lactobacillus plantarum 2s	191 glucose, 64 galactose
Streptococcus thermophilus 1t	362 glucose, 40 galactose

The *Lactobacillus plantarum* 2s strain produces an exopolysaccharide mainly composed of glucose monomers and of galactose. The exopolysaccharide produced by *Streptococcus thermophilus* 1t mainly contains glucose (**Table 2**). In *Lactobacillus plantarum* 2s, the glucose:galactose ratio is generally 3:1, corresponding to the data in the speciality literature. It must be emphasized that there is also the N-Acetylglucosamine, but it was not quantified in this study. For *Streptococcus thermophilus* 1t, the glucose quantity in the composition of the exopolysaccharide is 9 times larger. The existence of other compounds forming the polymer may be signaled here as well, but they were not determined either. In general, we noticed that the rest of the compounds (e.g. N-Acetylglucosamine) are present in a 1:1 ratio with the galactose quantity in the polysaccharide.

Conclusions

The fermentative studies show that, in case of conditions specific to the used strains, the production of exopolysaccharides is mainly associated to the logarithmic increase stage. The accumulation of exopolysaccharides is not associated to the logarithmical stationary stage. The results are comparable to other studies performed. What we notice is that the polysaccharide production is as good as when using the corn extract as a unique source of nitrogen, compared to the traditional sources. This finding is valid mainly for the *Streptococcus thermophilus* 1t strain. In exchange, for *Lactobacillus plantarum* 2s, we notice a decreased productiveness when the yeast extract is missing from the medium, as a traditional source of nitrogen. The corn extract cannot entirely substitute the lack of this ingredient. The composition of the polysaccharide is mainly made of glucose and galactose, in different ratios, depending on the strain. We noticed that, with both strains, glucose was majority, for *Lactobacillus plantarum* 2s the ratio was 3:1, and for *Streptococcus thermophilus* 1t – 9:1.

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