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Optimization of culture conditions for the production and extraction of *Klebsiella* sp. and *Pseudomonas* sp. bacterial exopolysaccharides

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ABSTRACT

Exopolysaccharides (EPS) are extracellular polysaccharides produced by prokaryotic and eukaryotic microbial cells, with a variety of practical applications in medicine and pharmacy, due to their excellent properties such as high solubility and biodegradability, as well as susceptibility to chemical modifications to obtain the desired features for specific applications. The aim of this study was to optimise the experimental conditions for the extraction of EPS produced by *Klebsiella* sp. and *Pseudomonas* sp. strains. The amounts of the exopolysachharides accumulated in different fractions of bacterial cultures, i.e bacterial whole culture, sediment, and supernatant proved to be quite similar. The glucose supplementation of the liquid growth medium stimulated the production of the exopolysaccharidic material by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains. The EPS material could be extracted with good yields by using a cost-effective method allowing to obtain sufficient amounts of dry material required for further characterization and conditioning in micro-sized formulations for drug delivery.

Keywords: *exopolysaccharides, Pseudomonsa aeruginosa, Klebsiella pneumoniae, extraction.*

1. INTRODUCTION

Microbial polysaccharides are a challenging subject of the current research field, with both theoretical importance in the understanding of their complex structural, physiological and host-pathogen relationships modulation roles and practical applications for the development of new anti-infective strategies, by achieving targeted controlled drug delivery systems with maximum therapeutic efficiency and minimal side effects.

Natural carbohydrates have a large variability in comparison with other macromolecules. In contrast to nucleotides and proteins, a monosaccharide is able to link, theoretically, to other monosaccharide in any position of the α-and β-bonds. If three nucleotides or amino acids can generate only six different combinations, three hexoses could produce between 1056 and 27648 different trisaccharides, and a hexose with six hexasaccharides could generate more than a trillion possible combinations [1].

Unlike polysaccharides and carbohydrate compounds isolated from plants and animals, where there have been identified about 20 monosaccharides, in the case of bacteria there have been purified by now more than 100 monosaccharides, generating an endless number of polymer combinations [2,3].

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Exploiting of bacteria producing exopolysaccaharides is an ongoing process and includes challenges for improving conditions for growth, genetic engineering and exploring fermentation substrata to reach low production costs [4, 5].

The aim of the study was to optimise and extraction method of bacterial exopolysaccharides produced by Gram-negative bacterial strains, belonging to *Klebsiella* sp. and *Pseudomonas* sp. genera.

2. EXPERIMENTAL SECTION

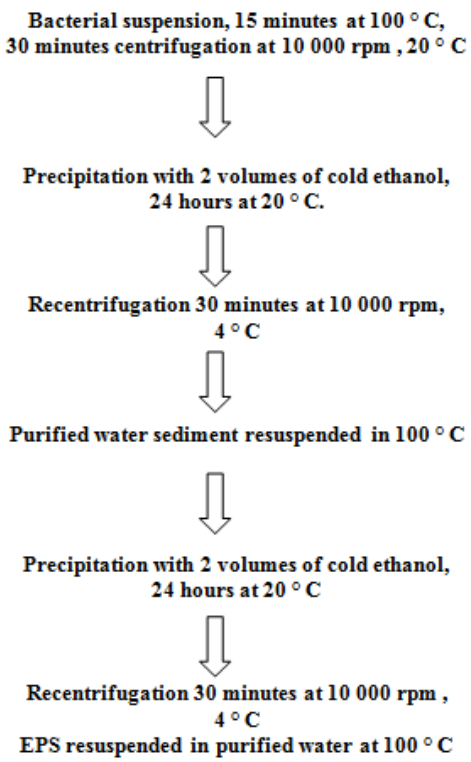


Figure 1: EPS extraction steps of EPS from bacterial *Klebsiella* and *Pseudomonas* culture fractions

2.1. The isolation of exopolysaccharidic substances (EPS) was performed from 27 strains of *Pseudomonas* sp. and 27 strains of *Klebsiella* sp. isolated from various pathological sources between 2009-2011, in the Bacteriology Laboratory of the Institute for Cardiovascular Diseases "Prof. Dr. C.C. Iliescu" and identified using API 20E and API 20NE microtest systems. After identification, the bacterial strains were included in the collection of the Microbiology Laboratory of the Botany-Microbiology Department of the Faculty of Biology, Bucharest.

2.2. The microscopic examination of capsular material was performed after negative staining with Congo red, using an optical microscope.

2.3. The isolation of bacterial EPS was conducted in the present study through a simplified method, adapted from Cerantola *et al.*, [6] (Figure 1).

2.4. The quantification of the extracted EPS was done from three different cellular culture fractions: cellular suspension, sediment and supernatant.

Bacterial suspensions were heated for 15 minutes at 100 °C and centrifuged for 30 minutes at 10 000 rpm. at 20 °C. Each variant was precipitated in a volume of 50 µL with 2 volumes of cold ethanol, for 24 hours at 20 °C. Thereafter, the working variants were recentrifuged for 30 minutes at 10 000 rpm at 4 °C, resuspended in hot purified water and precipitated with 2 volumes of cold ethanol, for 24 hours at -20 °C. After centrifugation for 30 minutes at 10 000 rpm at 4 °C, EPS fractions were resuspended in 50µL of hot purified water, which represented the final solution used to quantify the total sugar content by the phenol-sulfuric acid method [7] using glucose and sodium alginate as reference.

Quantification of total sugar concentration was achieved by measuring the absorbance value obtained at 492 nm using an ELISA reader.

The influence of culture conditions on the EPS production was studied for *Klebsiella pneumoniae* 742, *Klebsiella pneumoniae* 800 and *Pseudomonas aeruginosa* 568 strains in order to select the most appropriate experimental conditions to obtain the optimal amounts of EPS.

The selected strains were cultivated in casein soybean broth distributed in 300 mL Erlenmeyer glasses and inoculated with 1 mL bacterial suspensions corresponding to McFarland standard 1, with or without addition of glucose (50 g/L) and 20 g/L for *Pseudomonas aeruginosa* 568 and incubated with stirring at 250 rpm, at 30 - 35 °C. After incubation, the number of colony forming units was

determined and the produced EPS were quantified at different time points, i.e. 0, 2, 4, 8, 10, 24, 28 and 48 hours after inoculation.

The number of colony forming units was determined by plating a series of decimal dilutions mL of liquid medium cultured in casein soybean agar medium.

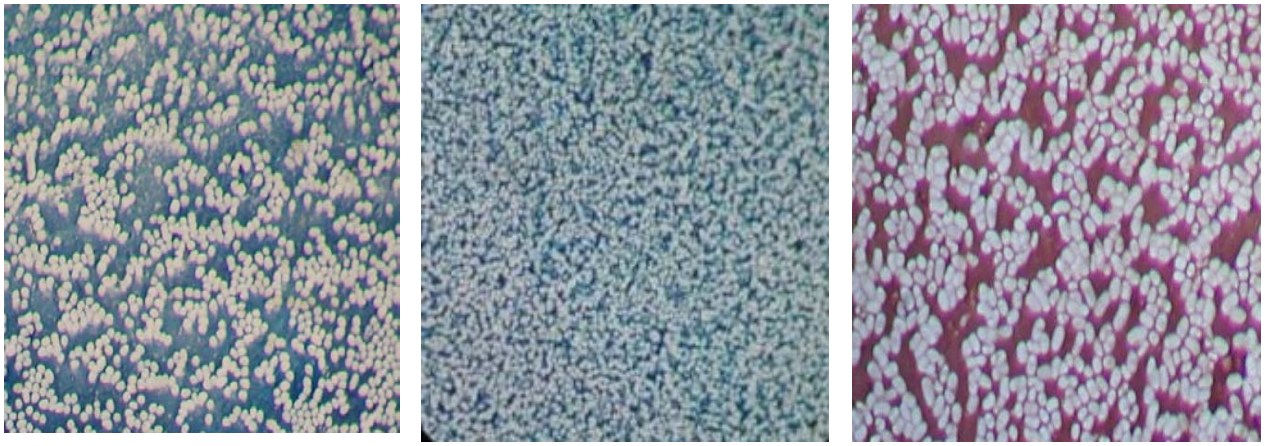
EPS were quantified as described previously, by measuring the absorbance at 492nm using an ELISA reader.

Further EPS purification was carried out from a culture volume of about 250 mL. The EPS fractions were obtained by cultivation in broth casein soybean, supplemented with 50 g/L glucose monohydrate for *Klebsiella pneumoniae* strains and 20 g/L glucose monohydrate for *Pseudomonas aeruginosa* strain at 30-35 °C, in conditions of stirring at 250 rpm, for 18-24 h.

In order to obtain an appropriate amount of biomass, 50 mL liquid medium were seeded in soybean casein agar distributed in 4 sterile polystyrene Petri dishes, 30x 30cm, incubated for 18-24 h at 30-35 °C. The biomass obtained after incubation was recovered, homogenized and after heat treatment for 15 minutes at 100 ºC, distributed into 50 mL centrifuge bowls of 200 mL capacity.

3. RESULTS SECTION

The optical microscopy images or Congo red stained microbial cultures are showing the ability of the selected strains to produce EPS, revealed by the occurrence of bright, non-colored structures contrasting with the dark colored microscopic field (figure 2).



P. aeruginosa 568 *K. pneumoniae* 742 *K. pneumoniae* 800

Figure 2: Microscopic images of Congo red colored smears performed from the selected bacterial strains cultures (1000x)

The scientific literature is citing different extraction methods of EPS from Gram-negative microbial strains cultures. For example, the EPS produced by *Klebsiella pneumoniae* was purified using the classical method of Westphal et al. [8], based on extraction with phenol and precipitation with methanol 1% (v/v). After holding at 20 °C, for 24 h, precipitates were centrifuged, recovered in distilled water and lyophilized. For further purification there was used digestion with nuclease (50 mg/mL of DNase and RNase II A) and proteinase K (50 mg/l). In order to remove trace amounts of LPS there was carried out ultracentrifugation at 105,000 g for 16 h at 4 °C, followed by freeze-drying [9, 10]. Cerantola *et al*., [6], isolated EPS from *Burkholderia cepacia* strains, grown on PIA (*Pseudomonas* Isolation Agar medium) with the addition of glycerol 2%, incubated for 3 days at 37 °C by centrifugation at 10 000 g for 30 minutes at 4 ° C.

During the present study we have used a method adapted from Cerantola *et al*.[6], as described above. The amounts of EPS obtained in the three experimental variants, i.e. suspension,

sediment, and supernatant were compared with two standards, represented by 1mg/mL solution of glucose monohydrate and sodium alginate (Sigma-Aldrich) (figures 3 and 4).

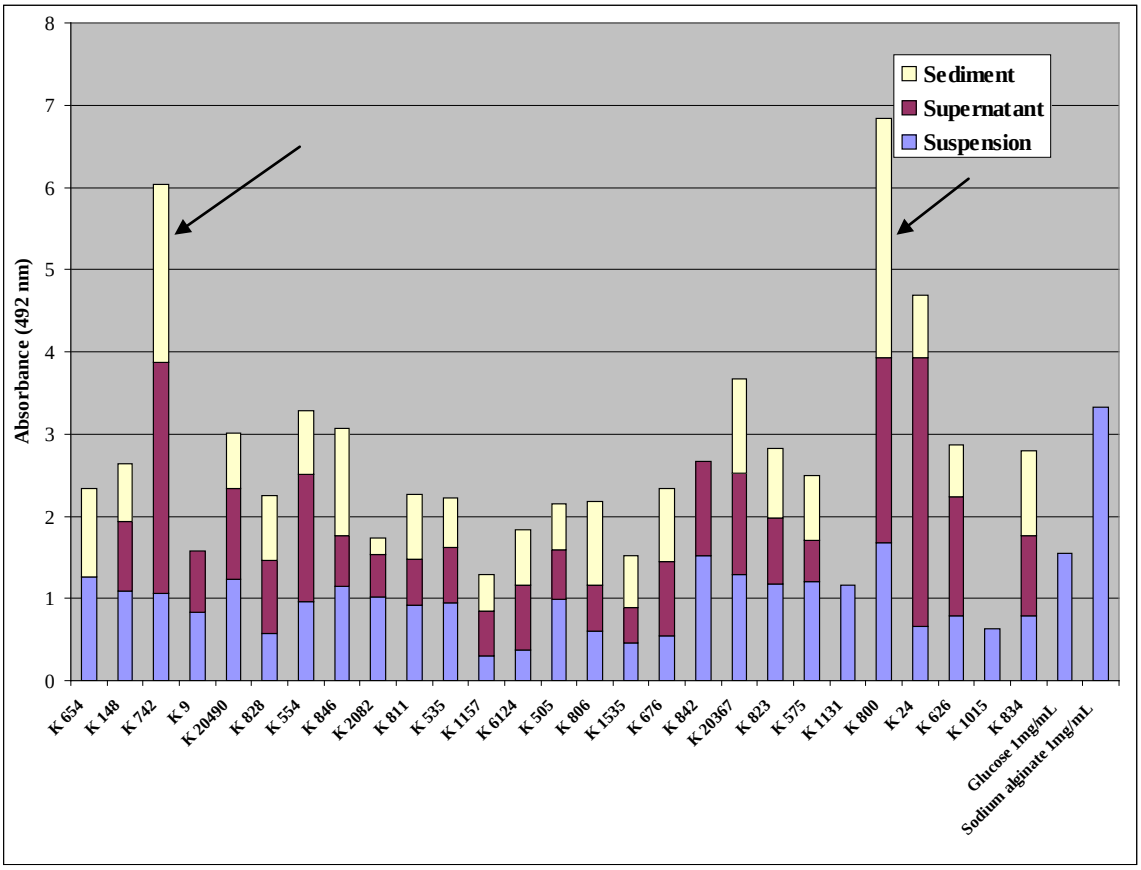


Figure 3: Absorbance values of different *Klebsiella* sp. culture fractions compared to the glucose monohydrate and sodium alginate control solutions at concentration of 1mg/mL

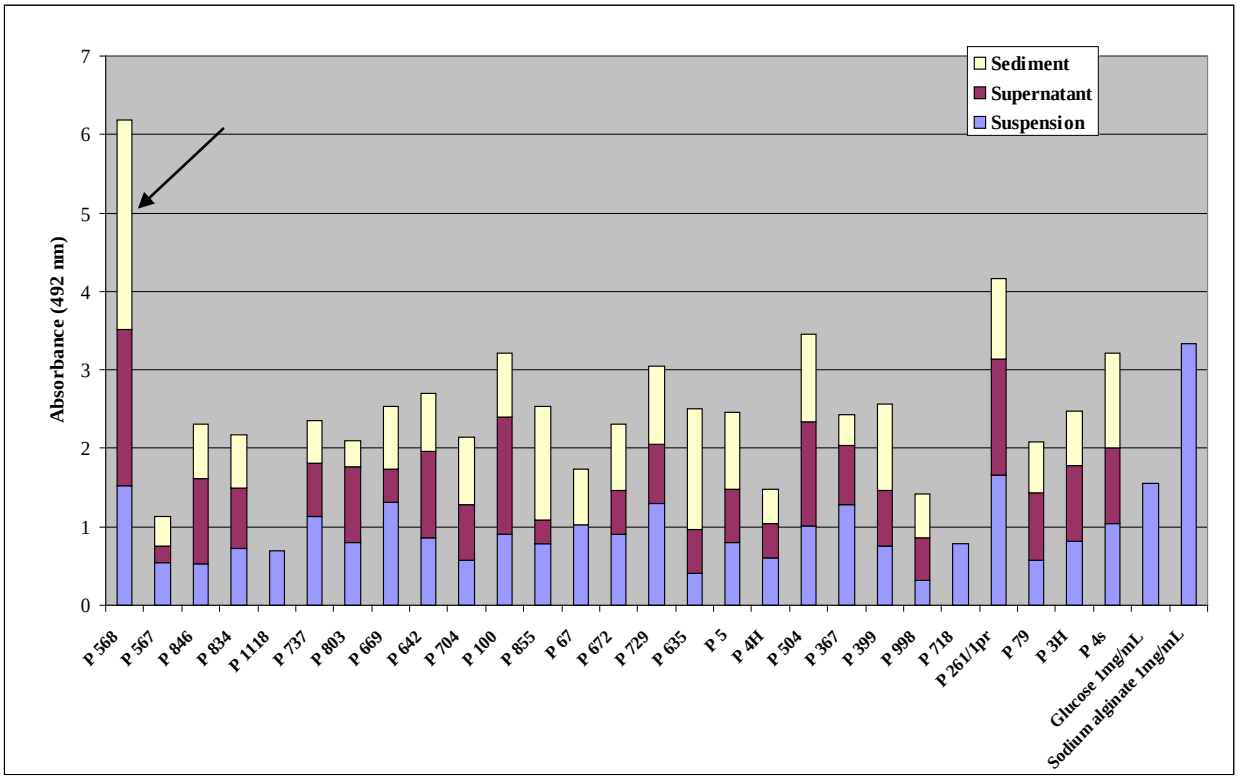


Figure 4: Absorbance values of different *Pseudomonas* sp. culture fractions compared to the glucose monohydrate and sodium alginate control solutions at concentration of 1mg/mL

There were no significant differences between the three analyzed fractions concerning the amount of microbial polysaccharides, excepting that the EPS were found in the suspension of all tested cultures, while in the case of the supernatant or sediments there have been cases in which the amount of EPS was below the method detection limit.

Based on the microscopy and EPS quantification results, *Klebsiella pneumoniae* 742, *Klebsiella pneumoniae* 800 and *Pseudomonas aeruginosa* 568 strains have been selected for high yields of EPS production polysaccharides. After selecting the three EPS producing bacterial strains, the quantification of capsular material in different cultivation conditions was performed, in order to determine the optimal cultivation parameters required for obtaining high yields of polysacharidic capsular material, appropriate for biotechnological applications.

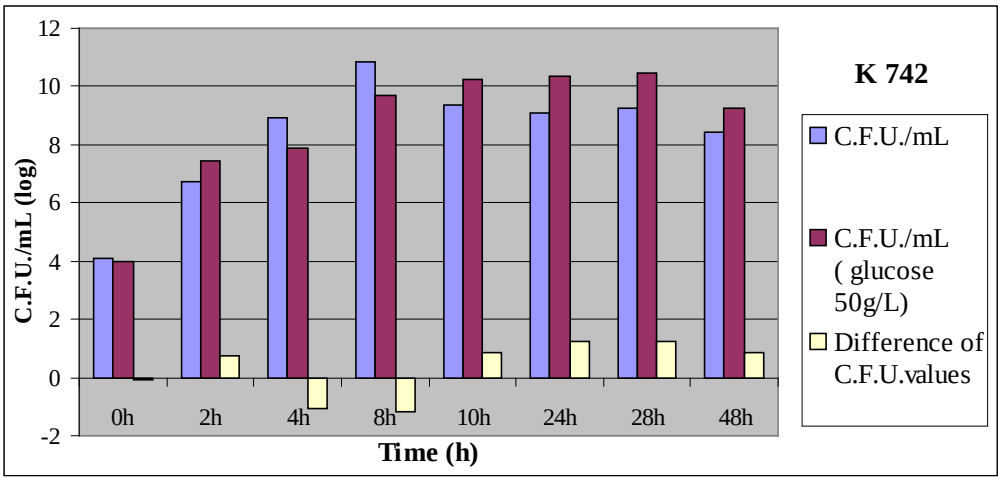


Figure 5: Graphical representation of the CFU/mL values of *Klebsiella pneumoniae* strain K742 obtained in the absence / presence of glucose

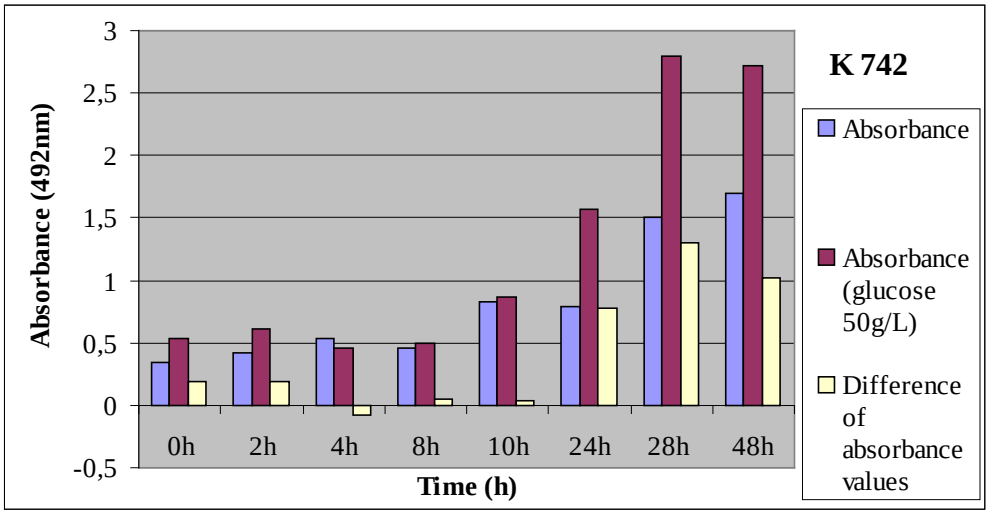


Figure 6: Graphical representation of the absorbance values of polysaccharidic fractions for *Klebsiella pneumoniae* strain K742 obtained in the absence / presence of glucose

The ability of microorganisms to produce EPS is determined by a direct response to the unfavorable natural environmental conditions [11, 12], but there is no direct evidence that an appropriate EPS-producing microorganisms selection could be based on the use of cultivation conditions similar to those of the natural environment. The properties and the EPS content is influenced by several factors which are represented by the composition of culture medium and the incubation conditions (temperature, pH, time) [13, 14, 15]. Stimulating microorganisms for the production of EPS may be

carried out in the presence of an improved carbon / nitrogen source [2]. Solid surfaces can stimulate the attachment of bacteria and the production of high amounts of EPS [16]. Alginate isolated from *Pseudomonas* sp. strains, attached to a Teflon mesh exhibited an increased activity of the gene algC compared to the unattached strains [17].

In the present study, the influence of glucose supplementation of the liquid growth medium (soybean casein) on the synthesis of the exopolysaccharidic material at 35 ° C, under stirring at 250 rpm was determined. The data were correlated with changes in the number of CFU/mL at different time points of the growth curve (0, 2, 4, 8, 10, 24, 28, 48 h of incubation).

Klebsiella pneumoniae strains K800 and K740 were cultivated in liquid medium supplemented with 50 g/L glucose monohydrate, and *Pseudomonas aeruginosa* strain P568 was cultivated in liquid medium supplemented with 20 g/L glucose monohydrate. For *Klebsiella pneumoniae* strain 742, glucose supplementation resulted in an increase in the number of CFU/mL of 1, 28 logs after 28 h of cultivation, correlated with the maximum value of absorbance of polysaccharidic fractions isolated from this strain (Figures 5, 6). For *Klebsiella pneumoniae* strain 800 the cultivation in glucose supplemented medium resulted in an increase in the number of CFU/mL of 1, 06 logs after 48 h of incubation (Figure 7), while the maximum absorbance values of the polysaccharidic fractions isolated from the strain K800 achieved the maximum values in presence of glucose after 28 h of growth (Figure 8).

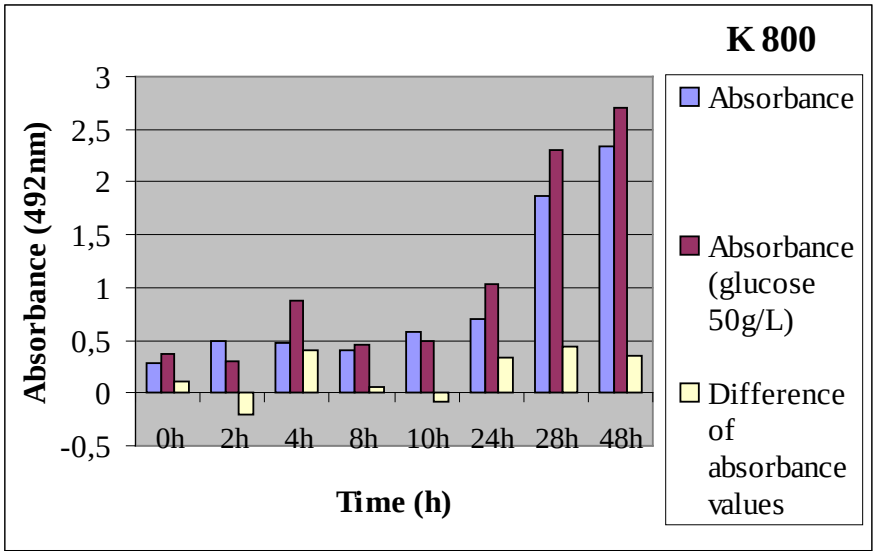


Figure7: Graphical representation of the CFU/mL values of *Klebsiella pneumoniae* strain K800 obtained in the absence / presence of glucose

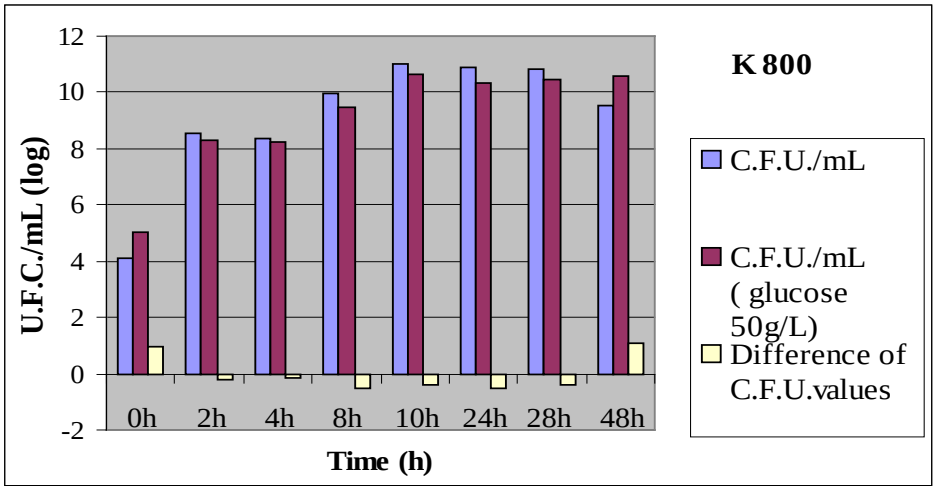


Figure 8: Graphical representation of the absorbance values of the polysaccharidic fractions of *Klebsiella pneumoniae* strain K800 obtained in the absence / presence of glucose

For *Pseudomonas aeruginosa* strain P568, the growth in glucose supplemented medium resulted also in an increase in the number of CFU/mL of 1, 42 logs after 24 h of cultivation (Figure 9), correlated with the maximum value of absorbance of polysaccharidic fractions isolated from the respective strain reached at 28h (Figures 9, 10).

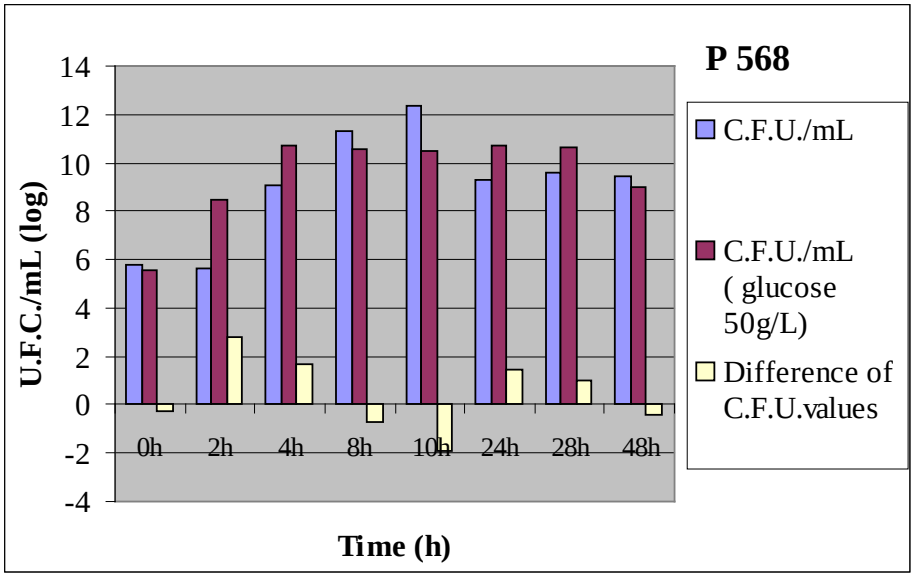


Figure 9: Graphical representation of the values of *Pseudomonas aeruginosa* strain P568 CFU / mL obtained in the absence / presence of glucose

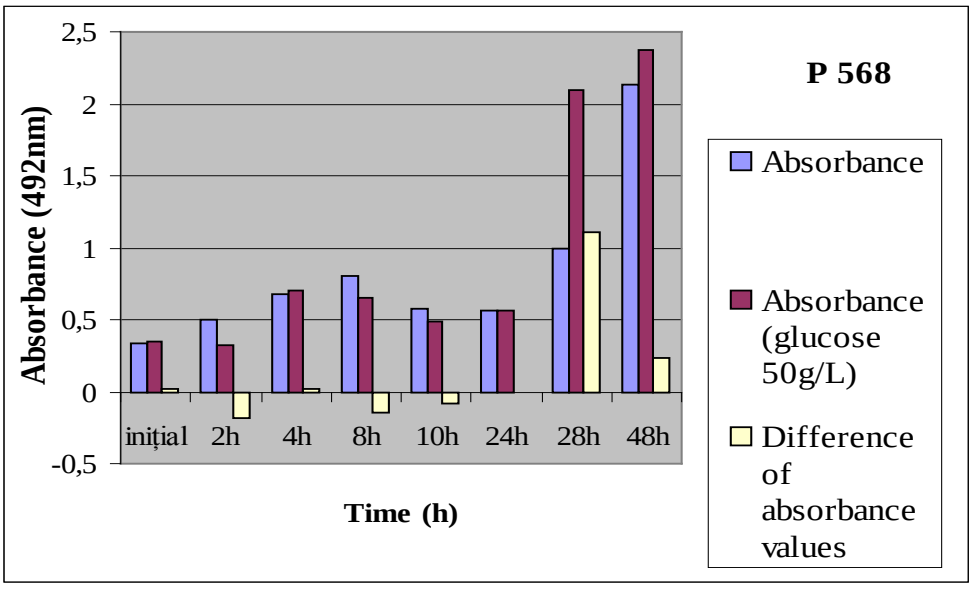


Figure 10: Graphical representation of the values of absorbance polysaccharidic fractions for *Pseudomona aeruginosa* strain P568 obtained in the absence / presence of glucose

In conclusion, the further EPS extraction was carried out from the strains K742, K800 and P568 in growth medium supplementation with glucose incubated under stirring conditions, at 35 ° C.

Purification of the EPS produced by selected bacterial strains was performed in a volume of approximately 250 mL liquid medium inoculated in three experiments carried out on three separate occasions. The subsequent steps of the extraction method respected Cerantola et al. modified method. The three polysaccharidic fractions obtained at the end of the extraction procedure were plated in sterile petri dishes and air-dried for about 48 h. Subsequently, the dried flakes of polysaccharidic material was mortared to obtain a powder of homogeneous appearance (figure 11).

Figure 11: The aspect of air-dried exopolysaccharidic material extracted from selected bacterial strains

After extraction there were obtained approximately similar amounts of exopolysaccharidic wet material and dry powder respectively from the three analyzed strains (table 1).

Table 1: EPS quantities of wet and dry material obtained after extraction

Bacterial strain	Experiement 1		Experiment 2		Experiment 3	
	Wet EPS	Dry EPS	Wet EPS	Dry EPS	Wet EPS	Dry EPS
K742	5.56	0.87	6.70	1.02	8.12	1.20
K800	7.32	1.47	6.43	0.98	6.89	1.19
P568	6.54	1.23	7.02	1.09	6.12	0.80

4. CONCLUSIONS

The amounts of the exopolysachharides accumulated in different fractions of bacterial cultures, i.e bacterial whole culture, sediment, and supernatant proved to be quite similar. The glucose supplementation of the liquid growth medium stimulated the synthesis of the exopolysaccharidic material by *Klebsiella pneumoniae* and *Paseudomonas aeruginosa* strains. The EPS material could be extracted with good yields by using a cost-effective method allowing to obtain sufficient amounts of dry material required for further characterization and conditioning in micro-sized formulations for drug delivery.

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