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Optimization of production of Microbial Exopolysaccharides (EPS) with essential oils from two medicinal plants

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ABSTRACT

Objective: The aim of our study is to evaluate the effect of essential oils of two medicinal plants: *Atriplex halimus* and *Haloxylon scoparium* on the production of EPS by four probiotic strains.

Methodology and results: Exopolysaccharides (EPSs) of lactic acid bacteria have potential for development and exploitation as food additives and functional food ingredients with both health and economic benefits. In this study, EPS production was carried at sucrose broth with different parameters (inoculum size, temperature, pH, incubation period, sucrose concentration, oxygen tension). In addition, under an optimized condition the effect of essential oils of two medicinal plants: *Atriplex halimus* (A.H.) and *Haloxylon scoparium* (H.S.) was evaluated on EPS production by four strains (*Leuconostoc sp., Lactobacillus sp*. and two strains *Pediococcus sp.).* The production kinetics and exopolysaccharide yields were strongly dependent on the fermentation conditions. Physical factors such as temperature, pH and oxygen tension had a primordial importance. Conditions leading to higher levels of EPS production depends on strains and adequate concentrations of sucrose (50 g/l), pH (6,5 for *Leuconostoc sp.* and *Pediococcus sp.,* 2; 4,5 for *Lactobacillus sp.* and 5 for *Pediococcus sp.* 1, incubation period (18 hours), temperature (37°C from *Leuconostoc sp.* and *Pediococcus sp.* 1, 30°C from *Lactobacillus sp.*, 50°C from *Pediococcus sp.* 2) and medium of production (broth sucrose). The introduction of essential oils (150 µl) of tested plants improved the production of EPS from 7.9 – 9.73 mg/ml of all strains to 15.2 – 16.28 with essential oils of *Atriplex halimus* and 11.6-13.2 mg/ml with essential oil of *Haloxylon scoparium*. Results showed an important stimulation of the exopolysaccharides production by essential oils of two plants (A.H. and H.S.)

Conclusion and Application of results: Our strains have shown their ability to produce maximum levels of EPS in the case of the addition of essential oils extracted from medicinal plants. These substances can be used as additives in the food industry to increase the productivity of the lactic strains of EPS as well as in the pharmaceutical field to prepare some medications.

Key words: exopolysaccharides, optimization, essential oils, medicinal plants, Lactic Acid Bacteria.

INTRODUCTION

Polysaccharides are the most diverse families of biopolymers. Many kinds of polysaccharides are found to have wide applications likes thickeners, stabilizers, emulsifiers, gelling agents and water binding agents in food industry, plasma substitutes, for skins or for transport of active ingredients in the medical field. Much attention has been focused on polysaccharides for their multiple bioactivities and pharmacological actions (Xiaohua and Lina, 2009). The primary structure of polysaccharides varies in composition and sequence, and different kinds of polysaccharides are characterized by regular repeating units. The bioactivity of a given polysaccharide is closely related to its structure and physicochemical properties (Xiaohua and Lina, 2009). Microbial exopolysaccharides are extracellular polysaccharides (EPS) produced by many microorganisms (Kumar *et al.*, 2007), mainly involved in cell adhesion and protection, and often covalently bound to the cell surface in the form of capsules, or secreted into the extracellular environment in the form of slime (Sivakumar *et al*., 2012). Lactic acid bacteria (LAB) produced EPS have been widely studied for their physicochemical properties and potential health effects during the last decades (Degeest *et al*., 2002) in view their application in food industry as thickeners, stabilizers, emulsifiers, binders, gelling agents as well as the beneficial physiological effects on human health, such as antitumour activity, immunomodulating bioactivity and antimutagenicity (Van Calsteren *et al*., 2002; Doleyres *et al*., 2005). The EPS are high molecular weight polymers, which are long chain composed of sugar residues and secreted by microorganisms into the surrounding environment. These molecules consist of a complex mixture of macro molecular polyelectrolytes including polysaccharides, proteins and nucleic acids. Each comprises of variable molecular mass and structural properties (Vijayabaskar *et al*., 2011). The majority of EPSs are heteropolysaccharides (HePSs) containing long chain repeating subunits of two or more monosaccharides and they can be generated in large quantities by microrganisms, such as dextran by *Leuconostoc mesenteroides*, *Streptococcus mutan*, *Gluconobacter oxydan*; Levan by *Lactobacillus reuteri* strain 121; and fructan by *Lactobacillus sanfranciscensis* LTH2590 (Van Geel-Schutten *et al.*, 1998; De Vuyst and Degeest, 1999; van Hijum *et al.*, 2001; Korakli *et al.*, 2002; Naessens *et al.*, 2005). The low production yield of these molecules represents a disadvantage for their uses in food industry, which makes development of new strategies for improved synthesis of EPS. In the present study, an attempt was made to optimize the production of exopolysaccharides by lactic acid bacteria. Process optimization was performed to maximize the production of EPS by addition of essential oils of two medicinal plants: *Atriplex halimus* (GUETTAF) and *Haloxylon scoparium* (REMTH).

MATERIALS AND METHODS

Lactic acid strains and culture medium: The lactic acid bacterial *Leuconostoc sp.* (S1)*, Lactobacillus sp.* (S2)*,* and 2 strains *Pediococcus sp* (S3 and S4) were isolated from cow milk, Faecal sample of infant (1 month) and fish respectively. All strains were cultured on anaerobiosis at 37 °C for 48 hours in Man Rogosa Sharp agar **(de Man *et al*. 1960),** and purified with cultures on MRS broth. The initial bacterial load ranged from 7 to 8 Log CFU/mL.

Medicinal plants: Two plants have been used: *Atriplex halimus* L. (A.H.) and *Haloxylon scoparium* Pomel (H.S.) collected from south west of Algeria, Mechria and Naâma respectively. The plants have dried away from light and moisture.

Extraction of essential oils: This operation was carried out in three main steps; hydrodistillation which is to bring to boil the mixture water and the dry plant: *Atriplex halimus* and *Haloxylon scoparium*. Then, from the distillate recovered from the previous step, organic phase were the essential oil must be removed from the aqueous phase with a solvent (cyclohexane). Finally, the organic material is placed in a rotary evaporator to recover the pure essential oil **(Willem, 2004).** Essential oils extracted were sterilized by filtration (0.45 µm).

Optimization of production of exopolysaccharides : Medium condition and other bacteria growth conditions are important factors for EPS production **(Xu *et al*., 2003; 2010; Kaditzky and Vogel, 2008; Hao *et al*., 2010).** This

step is to vary successively each of the factors keeping the other constant**.** Media (10 ml) were inoculated (1%) with strains pregrown in MRS broth (culture of 18 hours). The optical density (OD = 600 nm) is equal to a bacterial load of 2 × 10^8 CFU/mL

a. **Medium of production:** In order to evaluate the effect of medium on EPS production, the following media was tested: MRS and sucrose broth. Sucrose broth was prepared with sucrose 50 g/l, tryptone 10 g/l, Beef extract 5 g/l and KH_2PO_4 5 g/l , pH of medium was adjusted to 6,5.

b. **Concentration of inoculum:** The initial concentration of inoculum was varied as follows (10^6, 10^7,10^8), then incubation was carried out at 37 ° C for 24 hours.

c. **Temperature:** In order to determine the suitable temperature improved production of EPS, strains were grown at different temperatures: 30 °, 37 °, 45 ° and 50 ° C for 24 hours.

d. **pH:** To set the pH leading to optimal production of EPS, four different (3; 4.5; 5 and 6.5) were used. Cultures have incubated during 24 hours.

e. **Incubation period:** EPS production was investigated after incubation during 18, 24 and 48 hours.

f. **Conditions of aeroanaerobiosis:** The strains were cultivated in aerobiosis means growth in the presence of oxygen and anaerobiosis, which consists of life in the absence of oxygen under optimal conditions of the concentration of inoculum, pH, and temperature and incubation period.

g. **Substrate**: In the present experiment, strains were cultivated with different concentrations of sucrose: 30, 50, 70 and 90 g/l.

Effect of essential oils on EPS production: In order to evaluate the effect of essential oils of two plants (A.H. and H.S.) on EPS production, strains were grown in a medium supplemented with essential oils at different concentrations: 50, 100 and 150 µl respectively.

Kinetic of production of EPS: The kinetics of production were followed on broth sucrose under optimal conditions over a period of 72 h. EPS production was estimated at several time intervals (2 h, 4 h, 18 h, 24 h, 48 h, 72 h).

Isolation and quantification of exopolysaccharides : After incubation, the cultures were maintained in the water bath at 100°C for 10 min. Then they are cooled for 10 min at room temperature (25°C), treated with 85 % trichloracetic acid solution (v/v) and centrifuged at 13,000 rpm for 20 minutes **(Frengova *et al.,* 2000).** After removal of the cells and protein by centrifugation, the EPS was precipitated with ethanol (90 %). The EPS was recovered by centrifugation at 14,000 rpm for 20 minutes. Total EPS (expressed as mg/l) was estimated in each sample by phenol-sulphuric method **(Dubois *et al.,* 1956)** using glucose as standard **(Torino *et al.,* 2001).**

***In vivo* test (measure of viscosity):** To evaluate the effect of stem strains *in vivo*, skim milk was inoculated with the strains (S1, S2, S3 and S4) at 1% volume **(Dupont, Roy, & Lapointe, 2000).**

Statistical analysis: All experiments were repeated in duplicate. All data were presented as means ±SE. Frequency of isolated strains was examined for statistical significance using analysis of variance (ANOVA) (Fisher's test). A p value of 0.05 was considered as the threshold to declare a statistically significant difference.

RESULTS AND DISCUSSION

Yield of extraction of E.O: Yields of extraction were 0.028 what should be the result of Chikhi I., 2013 for *Atriplex halimus* and 0.019 % for *Haloxylon scoparium*. Otmani F., 2014 have found a performance of extraction of essential oil of Haloxylon scoparium harvested from Naâma of 0.019 %.

Optimization of production of EPS: The results from initial assays indicated that the ideal conditions for the synthesis of EPS by strains were:

a. **Medium of production:** Figure 1 (a) indicated that variable amounts of EPS could be achieved using different media. Relatively low EPS yields were maintained using MRS, however, the maximum EPS yield was recorded in case of sucrose broth. Similar results were obtained by Khadem H. *et al*, 2012. As the composition of the growth medium has an important influence on EPS production (Cerning *et al*., 1994).

b. **Concentration of inoculums:** As showed in figure 1 (b) the highest yield of EPS was collected with 10^7 UFC/ml. Results revealed a proportional relationship between initial inoculum concentration and EPS production under optimal value. Similar results were obtained by Amar Yacine *et al*., 2012.

c. **Temperature:** The optimal temperature for EPS synthesis was 30°C for the strains *Lactobacillus rhamnosus* and *Pediococus damnosus* (1), 37°C for *Leuconostoc* mesenteroïdes and 50°C for *Pediococcus damnosus* (2) as shown in Figure 1(c). Leo *et al*., 2007 indicated that *Lactobacillus fermentum* TDS030603 is able to produce more EPS at 37 than at 40°C. On the other hand, the growth and exopolysaccharide (EPS)

production by *Lactobacillus fermentum* F6 increased to a maximum value of 44.49 mg/l of EPS at 37°C (Zhang *et al.*, 2011).

d. **pH:** pH is one of the most important factors, which can influence growth, and production of particular products by lactic acid bacteria. In this study, the final rate of EPS was higher at pH6.2 for all strains except the strain *P. damnosus* (S4) to pH 5. Therefore, pH 6.5 and 5 for *P. damnosus* (S4) was chosen for the rest of the study (Fig 1d). Bakry M. Haroun *et al.*, 2013 results revealed that the maximum level of EPS produced by *Lactobacillus plantarum* was reached at pH 6.2.

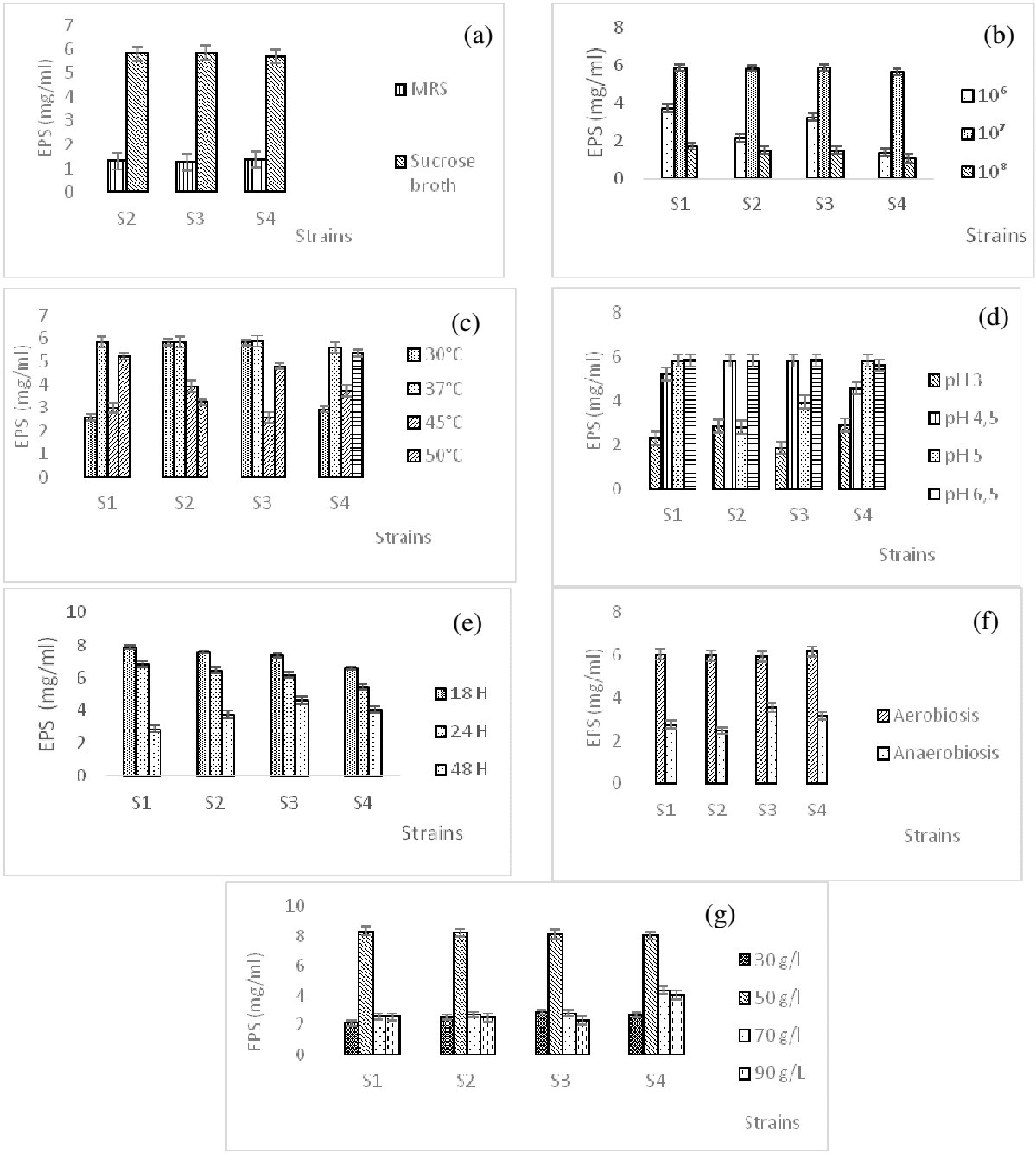

Figure 1: Effect of differents factors on EPS production: (a): medium of culture, (b): inoculum size, (c): temperature, (d): pH, (e): incubation period, (f): aeroanaerobiose, (g): concentration of substrate [P(0,05)]

e. **Incubation period:** Results illustrated in figure n 1(e) indicate that biosynthesis of EPS was increased linearly to reach its maximal yield after 18 h. Above this phase of growth; a decline in the EPS production was recorded, until a depletion of EPS production was observed at 48 h. The most of EPS was produced during the 12 to 24 h incubation of the fermentation (Richard and Maria, 2003).

f. **Conditions of Anaerobiosis:** Results revealed that oxygen amount affects EPS production. Figure

g. 1f showed that EPS production was maximal at aerobiosis for all strains. Studies of Amar Y. *et al*., 2012 and Khadem H. *et al*., 2012 reported that the highest level of EPS was obtained at aerobiosis.

h. **Substrate:** It has also been hypothesized that bacterial growth and EPS production are usually influenced by initial concentration of carbon (Wachenheim and Patterson, 1992). In this experiment, among the various concentrations tested, 50 g/L was the most suitable for EPS production for all strains in concordance with research of Amar Y. *et al.,* 2012 (Fig 1g). It has been noted that this parameter significantly affected the production of EPS.

Effect of essential oils of two plants on EPS production

Effect of E.O. from two plants on EPS production: The potential of essential oils for the production of EPS by the lactic strains was determined in this experiment. This study results (Fig. 2) showed that essential oils from two plants (A.H. and H.S.) stimulate selectively the EPS production where there was an increase in EPS level with increase of oils concentration. The higher production was obtained with 150 µl for all strains.

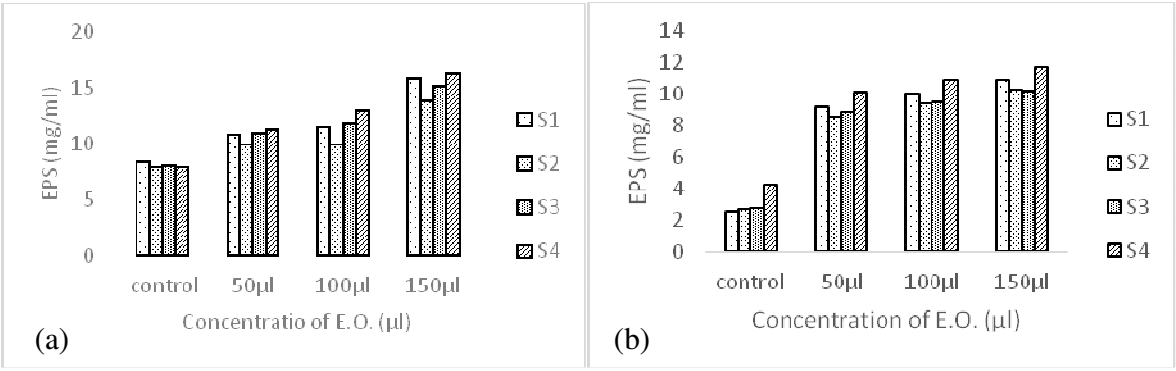

Figure 2: Effect of essential oils of medicinal plants on EPS production. (a): E.O. of *Atriplex halimus*, (b): E.O. of *Haloxylon scoparium*

Kinetic of EPS production: The results presented in Figure 3 indicated that the addition of essential oils of plants (A.H. and H.S.) improved the production of EPS by studied strains but the kinetics of production were same with and without essential oils. However, the highest production of EPS was observed with the strain *Pediococcus* 2 (S4). EPS was produced mainly during the exponential growth phase and continued slightly in the stationary phase. Similar observations were reported for other EPS-producing LAB strains (Duenas *et al*., 2003). Many studies showed a decrease in the total EPS amount upon prolonged fermentation. The decrease is difficult to explain since viscosity measurements may be affected not only by the amount of polysaccharides released by the cells, but also by other metabolic products such as lactic acid and proteins (Walling *et al.,* 2005). However, growth associated and non-growth-associated production of EPS by LAB has been observed before (Manca de Nadra *et al*., 1985; Kojic *et al*., 1992; Looijesteijn and Hugenholtz, 1999).

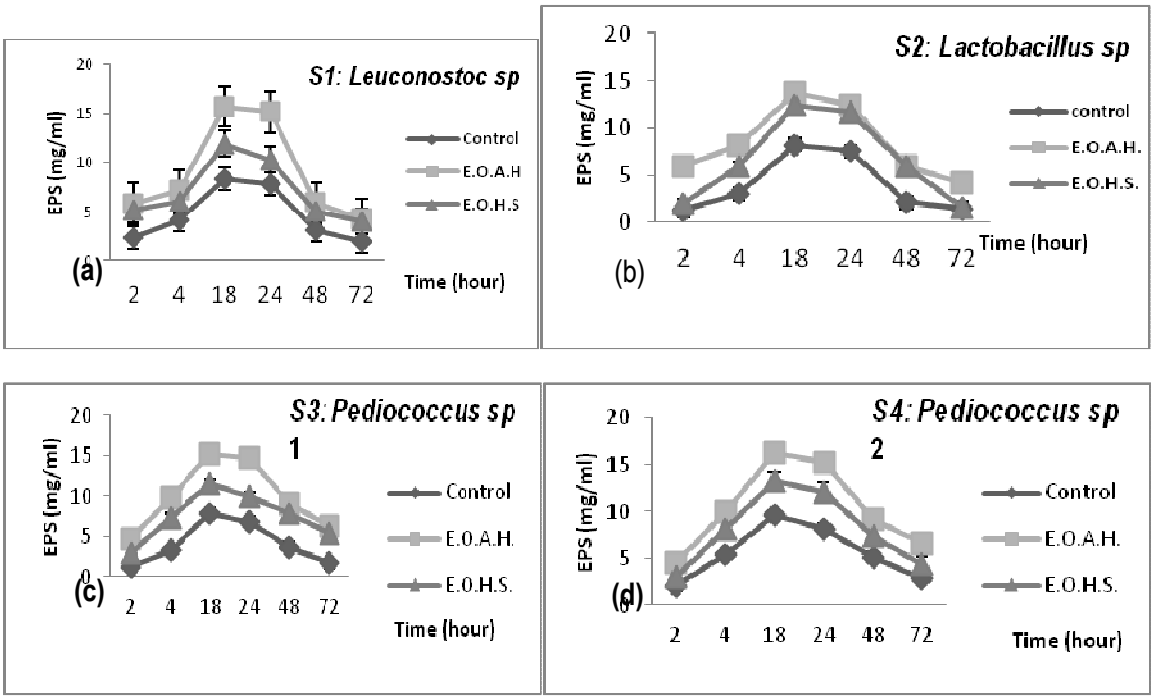

Figure 3: Impact of E.O. from two medicinal plants on kinetics of EPS production in each strain

***In vivo* test (Viscosity test):** There was an obvious change in viscosity during the fermentation period, indicating that EPS production had occurred. On the other hand, the study found an increase in viscosity when essential oils were added to cultures of strains (Figure 4). Moreover *Pediococcus damnosus* 2 (S4) represent the excellent strain in terms of improving viscosity. Exopolysaccharides produced by some strains of lactic acid bacteria have been found to improve textural properties in fermented dairy products (Ruas-Madiedo *et al.,* 2002).

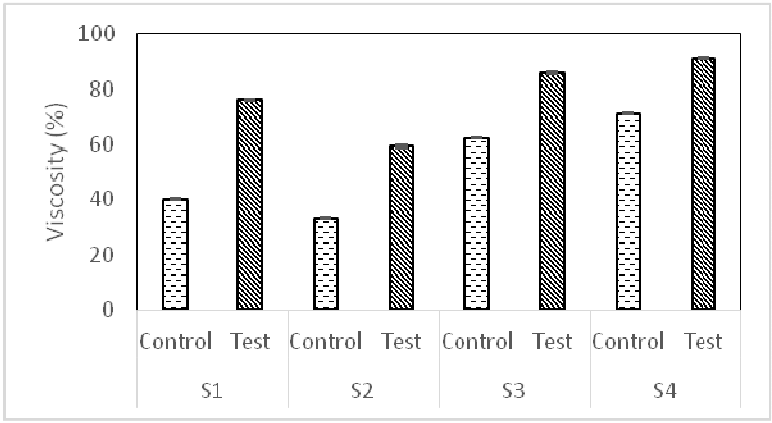

Figure 4: viscosity of skim milk inoculated with different strains

CONCLUSION AND APPLICATION OF RESULTS

This study showed that the strains have their ability to produce extracellular polymers. The essential oils of two plants *Atriplex halimus* and *Haloxylon scoparium* may exert a selective stimulation on EPS production. The results from this study revealed that some substances, such as essential oils, could be used as a substitute substrate for the EPS production. Although the obtained results do not allow direct extrapolation, they are an indication of the possible ability of essential oils to be used to optimize EPS production. In vivo studies are

needed to confirm the optimizing effect of essential oils of medicinal plants from the production of exopolysaccharides.

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