GIDA (2014) 39 (4):195-202 *Research* / Araştırma

EXOPOLYSACCHARIDE PRODUCER *STREPTOCOCCUS THERMOPHILUS* ST8.01 STRAIN; A POTENTIAL PROBIOTIC CULTURE

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Received / Geliş tarihi: 17.02.2014
Received in revised form /Düzeltilerek Geliş tarihi: 21.03.2014
Accepted / Kabul tarihi: 24.03.2014

Abstract

The aim of this study was to determine the probiotic potential of exopolysaccharide (EPS) producer *Streptococcus thermophilus* ST8.01 strain. This strain was able to survive at pH 3 and 1% bile salt. Viable counts were enumerated as 4.80±0.04 and 2.11±0.06 log cfu/mL after exposure to 0.4% phenol and gastric juice at pH 3, respectively. *Strep. thermophilus* ST8.01 was able to grow at 100 mg/L lysozyme and showed to have high autoaggregation (49.55±6.24%) and hydrophobicity abilities (67.23±7.16%). The ST8.01 strain was also found sensitive to most clinically important antibiotics. Results obtained in this study suggest that *Strep. thermophilus* ST8.01 strain may be used as a probiotic starter culture to produce dairy products.

Keywords: *Streptococcus thermophilus,* exopolysaccharide, probiotic properties, antibiotic susceptibility

EKZOPOLİSAKKARİT ÜRETİCİSİ *STREPTOCOCCUS THERMOPHILUS* ST8.01 SUŞU; POTANSİYEL PROBİYOTİK KÜLTÜR

Özet

Bu çalışmanın amacı, ekzopolisakkarit (EPS) üreticisi *Streptococcus thermophilus* ST8.01 suşunun probiyotik potansiyelinin belirlenmesidir. Bu suş pH 3 ve %1 safra tuzunda hayatta kalma yeteneğine sahiptir. %0.4 fenol ve pH'sı 3'e ayarlanmış mide suyu uygulaması sonrası canlı hücre sayısı sırasıyla 4.80±0.04 ve 2.11±0.06 log kob/mL olarak ölçülmüştür. *Strep. thermophilus* ST8.01, 100 mg/L lizozim konsantrasyonunda gelişebilme ve yüksek otoagregasyon (%49.55±6.24) ve hidrofobisite (%67.23±7.16) yeteneğine sahiptir. ST8.01 suşu aynı zamanda klinik olarak önemli olan antibiyotiklere karşı duyarlı bulunmuştur. Bu araştırmadan elde edilen sonuçlar, *Strep. thermophilus* ST8.01 suşunun süt ürünleri üretiminde probiyotik starter kültür olarak kullanılabileceğini düşündürmektedir.

Anahtar kelimeler: *Streptococcus thermophilus,* ekzopolisakkarit, probiyotik özellikler, antibiyotik duyarlılık

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INTRODUCTION

The relation between nutrition and good health has become more interesting during the recent years. Researches have increased to defining of food and food components with this driving force of increasing conscious of population. Probiotic products were defined as foods that contain live microorganisms providing positive effects on health of consumers (1). Especially popularity of dairy products with probiotic bacteria have increased day by day and still be demanded on the functional food market (2). Briefly probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" by WHO/FAO (3).

Lactic acid bacteria, particularly the *Lactobacillus* and *Bifidobacterium* spp. are known as probiotics most extensively studied and widely used in dairy products. Besides these species, member of the genera *Enterococcus, Streptococcus* and *Propionobacterium* are also esteemed as probiotics (4). *Strep. thermophilus* is a common dairy starter extensively used in the production of yogurts, cheese and some fermented milk products. *Strep. thermophilus* which is recognized as safe (GRAS), can survive in the gastrointestinal system; even though it is known that the ability to attach to intestinal epithelial cells is relatively weak and it could be damaged in gastric acidic condition (5-7). Also it has some inherent functional properties important for food industry such as organoleptic, technological and nutritional advantages (8). Besides these beneficial effects it is indicated that *Strep. thermophilus* can promote the health of human and animals. Different researchers reported that *Strep. thermophilus* can improve the intestinal microflora, prevent diarrhea induced by using antibiotics, reduce the lactose intolerance and the risks of certain cancers, ulcers, imflammation, stimulate the immune system, and also it can be used for curing some atopic dermatitis (9-17). In addition many *Strep. thermophilus* strains have most of the probiotic characteristics (resistance to bile salts, low pH degrees and gastric juice, hydrophobisity activitiy, etc.) desired for starter culture development processes (18, 19).

The goal of this study was to determine some probiotic properties and antibiotic susceptibility of *Strep. thermophilus* ST8.01 which was characterized as exopolysaccharide (EPS) producer previously (20) isolated from homemade yogurt.

MATERIAL and METHODS

Bacterial Strain and Growth Condition

Streptococcus thermophilus ST8.01 strain used in this study was previously isolated from homemade yoghurt and characterized as EPS producer (20). Strain ST8.01 was grown in M17 broth (Merck KgaA, Darmstadt, Germany) with 5% glucose (GM17) during the experiments. Stock culture of the strain was maintained at -20 °C in GM17 broth added with 15% glycerol.

Bile Salt Resistance and Acid Tolerance

Ability of tolerance for bile salts was determined based on the method of Gilliland and Walker (21) with slight modifications. *Strep. thermophilus* ST8.01 was inoculated (1 %, v/v) in GM17 broth supplemented with 0.3%, 0.5% and 1% (w/v) ox bile (Acumedia, Lansing, Michigan, USA) and without ox bile as a control incubated at 37 °C. Survival of the strain was determined on the GM17 agar as colony forming units (cfu) at 0. h and 24. h. Inhibition (%) was calculated with the following equation:

$$Inhibition\ \% = \{[(initial\ cfu/mL) - (final\ cfu/mL)] / [initial\ cfu/mL]\} \times 100 \quad (1)$$

For detecting of low pH tolerance, the pH of the 2 mL volume of sterile phosphate-buffered saline (PBS) was adjusted to 1, 3 and 5 with 1M HCl and pH 7.2 as control. Overnight culture of ST8.01 strain was harvested by centrifuged at 3000 g (10 min, 4 °C) and washed once in sterile PBS. Pellet resuspended into PBS one-tenth of the culture volume was centrifuged. The 0.1 mL of suspension was added into 2 mL PBS at pH 1, 3 and 5 then incubated at 37 °C. Viable cells were enumerated at 0., 1., 2., 3. and 4. h on GM17 agar plates (22).

Tolerance to Simulated Gastric Juice

Sterile saline solutiun (0.5%, w/v) with pepsin (Sigma-Aldrich, St Louis, Missouri, USA) (0.3%, w/v) adjusted to pH 2 and 3 were used to simulate gastric condition. 30 mL overnight culture was centrifuged at 6000 g at 5 °C for 20 min. Pellet was washed twice with K_2HPO_4 (50 mM, pH 6.5) and then resuspended in 3 mL of the same buffer. 1 mL of resuspended culture was centrifuged at 12000 g (5 min, 5 °C) and resuspended in gastric juice (10 mL) pH 2 and 3. At the begining of incubation period and at the end of 3 h incubation

time viable cell counts were performed on GM17 agar (18). Inhibition (%) was calculated with equation 1.

Resistance to Lysozyme and Survival in The Presence of Phenol

The effect of lysozyme on the growth on *Strep. thermophilus* ST8.01 was examined by the method of Brennan et al. (23). The activated culture was inoculated (2%, v/v) into GM17 broth with and without 100 mg/L lysozyme (Sigma-Aldrich). Bacterial cells were counted on GM17 agar plates at 0., 3. and after 24 h incubation at 37 °C. Survival of the *Strep. thermophilus* ST8.01 in the presence of phenol was determined based on the method of Teply (24). Overnight culture was inoculated (2%) into 10 mL GM17 broth with and without 0.4% phenol and incubated at 37 °C. Viable cell numbers were determined at 0., 3. and 24. h of incubation on GM17 agar plates. Inhibition (%) was calculated with equation 1 for phenol treatment. Increase (%) was calculated with equation 2 for lysozyme treatment:

$$Increase\ \% = \{[(final\ cfu/mL) - (initial\ cfu/mL)] / [final\ cfu/mL]\} \times 100 \quad (2)$$

Autoaggregation Assays

Overnight culture was harvested by centrifugation at 7000 g (10 min, 20 °C) and washed with sterile saline (0.85% NaCl, w/v). Cell consantration was adjusted $A_{660\ nm}$ = 0.3 within sterile saline and incubated at 37 °C for 60 min. The end of the incubation period suspension was centrifuged at 300 g (2 min, 20 °C) and absorbance of the supernatant (A_{60}) was measured (25). Autoaggregation was calculated with the following equation:

$$Autoaggregation = (A_0 - A_{60}) / A_0 \times 100 \quad (3)$$

Hydrophobicity

Bacterial culture was centrifuged at 5000 g for 15 min after growth in GM17 broth overnight. Pellet was washed twice in sterile PBS and resuspended in KNO_3 (0.1 M, pH 6.2) then absorbance of the cell suspension was adjusted at A_0 = 0.5-0.6 (600 nm). 1 mL of xylene was added to cell suspension and incubated at room temperature for 10 min. After this preincubation time suspension was vortexed (2 min) to mix the two phase system and incubated at room temperature. At the end of the 20 min, aquase phase was removed carefully and its absorbance was measured at 600 nm (A_1) (26). The percentage of cell cerface hydrophobicity was determined using the following equation:

$$Hydrophobicity = 1 - (A_1 / A_0) \times 100 \quad (4)$$

Antibiotic Susceptibility

Disc diffusion method was used to determine antibiotic susceptibility of Strep. thermophilus ST8.01 against vancomycin (30 µg, Oxoid Ltd, Wade Road, Basingstoke, Hants, UK), tetracycline (30 µg, Oxoid), streptomycin (300 µg, Oxoid), penicillin G (10 units, Oxoid), erythromycin (15 µg, Oxoid), ampicillin (10 µg, Oxoid), chloramphenicol (30 µg, Oxoid), gentamicin (120 µg, Oxoid), norfloxacin (10 µg, Oxoid) and sulphamethoxazole/trimethoprim (1.25+23.75 µg, Oxoid). Inhibition zones were measured as diameter (mm) and results were expressed as susceptible, moderate susceptible and resistant by comparing with the interpretative zone diameters given by Clinical and Laboratuary Standards Institute (27).

RESULTS and DISCUSSION

Bile Salt Resistance and Acid Tolerance

Bile tolerance has been described as an important factor for the survival and growth of LAB in the intestinal tract (21). Probiotic strains must be resistant to bile salt at 0.3% if these are to be used for human beings (28). In our study *Strep. thermophilus* ST8.01 maintained the viability at three concentrations of bile salt (0.3%, 0.5% and 1%, w/v) after 24 h incubation. The highest inhibition percentage of this strain was detected as 38.34 at 1% bile salt (Figure 1). Similar to our results, Iyer et al. (29) showed that two *Strep. thermophilus* strains survived at 0.5%, 1% and 2% bile salt concentrations after 180 min. Conversely Vinderola and Reinheimer (18) informed that bile resistance of *Strep. thermophilus* strains was poor

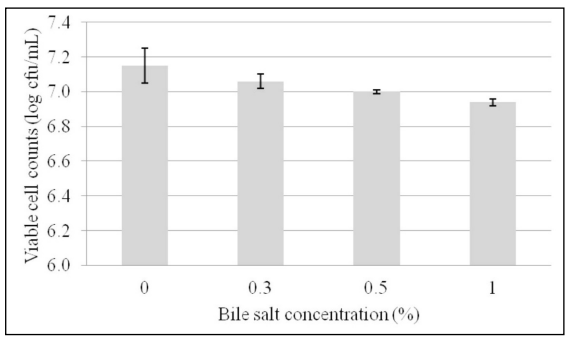

Figure 1. Effect of bile salt concentrations on the viability of *Strep. thermophilus* ST8.01 at 37 °C for 24 h.

and most of the strains were inhibited at 0.5% bile salt. In addition some researchers indicated that many *S. thermophilus* were inhibited at 0.15% bile salt (30). Contrary of this statement *Strep. thermophilus* ST8.01 survived even after 24 h at 1% bile salt as specified by Mahmood et al. (31).

The growth of *Strep. thermophilus* ST8.01 rapidly inhibited at pH 1 at the begining of the incubation and at the end of the two hours ST8.01 was completely inhibited. pH 3 represented less lethal environment to *Strep. thermophilus* ST8.01 than pH 1 but still cell viability was decreased during incubation and inhibition percentage of this strain was greater than >99.99% at pH 3. However, after 4 h at pH 5 *Strep. thermophilus* ST8.01 maintained its viability and the inhibition was detected at least (95.43%) for pH 5 among the low pH treatments (Figure 2). Previous studies showed that some Strep. thermophilus strains were dead when exposed to low pH degrees similar with our results (31-34). On the other hand Aswathy et al. (28) reported that most of the lactic acid bacteria isolates including *Streptococcus* grew at pH 5 and played a role during the fermentation of dairy milk and vegetables.

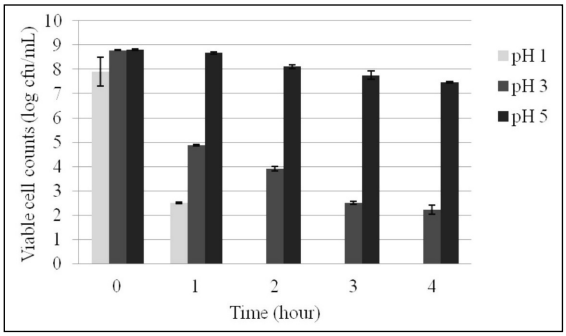

Figure 2. Effect of low pH on the viability of *Strep. thermophilus* ST8.01 at 37 °C.

Tolerance to Simulated Gastric Juice

Gastric juice studies were done in 3 hours incubation for implementing residence time in stomach condition. Among the lactic acid bacteria *Strep. thermophilus* was known to show more sensitivity to simulated gastric juice (18). *Strep. thermophilus* ST8.01 showed significant decrease in the viable counts at pH 2 and pH 3 with 0.3% pepsin after 3 h incubation at 37 °C (Figure 3). For simulated gastric juice experiments at pH 2 and pH 3, inhibition percentage of this strain were calculated as >99.99%. Similar to our results, Vinderola and Reinheimer (18) reported that *Strep. thermophilus* showed to have very poor survival under simulated gastric conditions. Controversially, Pilar et al. (35) and Iyer et al. (29) reported that *Strep. thermophilus* maintained the viability under the simulated gastrointestinal stress condition. On the other hand the evidence of viable *Strep. thermophilus* in human feces and so the transit from the gastrointestinal tract were observed from the volunteers consuming the yogurt samples (6, 7). Based on these data and maintanence the vitality of the strain ST8.01 (2.11 log cfu/mL) when exposed to pH 3 with 0.3% pepsin suggested that it may survive when consumed with fermented milk product such as yogurt but to prove that food applications should be done at the further studies.

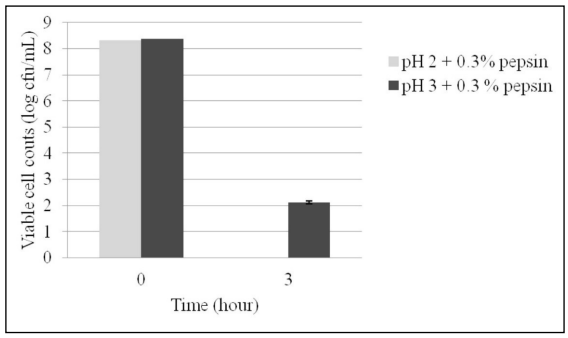

Figure 3. Tolerance to simulated gastric juice of *Strep. thermophilus* ST8.01 at 37 °C.

Resistance to Lysozyme and Survival in The Presence of Phenol

In this study we determined that *Strep. thermophilus* ST8.01 was grown even presence of lysozyme at the level of 100 mg/L (Figure 4). Increase percentages of control sample were calculated as 83.78% and 99.55% after first 3 h and 24 h incubation, respectively. For lysozyme experiment, increase percentages were exhibited as 69.80% and 95.32% after first 3 h and 24 h incubation, respectively. Lysozyme is used as attractive preservative to inhibit food spoilage bacteria which are harmful to human health. It is reported that using lysozyme at the levels up to 25 mg/L does not affect the growth of *Streptococci* and *Lactobacilli*. Vinderola et al. (36) determined that growth of the probiotic bacteria was not effected by the lysozyme treatment at the level of 25 mg/L and reported that high levels of lysozyme should be used for selecting probiotic starter bacteria.

On the other hand Xanthopoulos et al. (30) reported that some *Strep. thermophilus* showed resistance to 100 mg/L lysozyme.

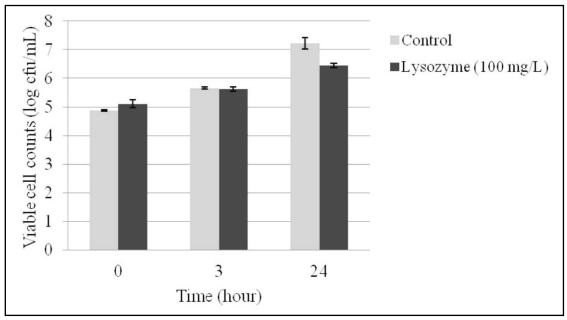

Figure 4. Resistance to lysozyme of *Strep. thermophilus* ST8.01 at 37 °C.

Phenols can be occured in the intestines because of the deamination of some aromatic amino acids from the digested foods through the agency of the bacteria. Therefore tolerance to phenols is a desired probiotic characteristic (21, 37). In the present study, growth of the *Strep. thermophilus* ST8.01 inhibited after 24 h presence of 0.4% phenol but at the end of the first 3 hours of incubation vitality of the strain ST8.01 was still retain (Figure 5). After 3 hours incubation, inhibition percentage of phenol treated sample was calculated as 64.52%. Different researchers reported that some lactic acid bacteria showed high tolerance to phenol (0.2-0.5%) (38, 39) but *Strep. thermophilus* was known as very sensitive to this chemical (30).

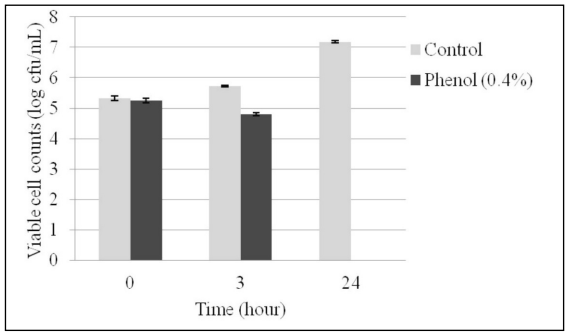

Figure 5. Resistance to phenol (0.4%) of *Strep. thermophilus* ST8.01 at 37 °C.

Autoaggregation Ability

Autoaggragation value of *Strep. thermophilus* ST8.01 was recorded as 49.55±6.24%. Among LAB various autoaggregation values were determined and it was indicated that the autoaggragation ability might be strain dependent. It was also reported that physochemical characteristics of cell surface (such as hydrophobicity) may affect the autoaggregation ability (40-42). The importance of autoaggregation ability for probiotics is that it might be necessary for their adhesion to the intestinal epithelial cells (43, 44). Based on the previous studies of Canzi et al. (45), Rahman et al. (46), and Kõll et al. (47), *Strep. thermophilus* ST8.01 has moderate autoaggregation but still this value might be considered to be higher then many of lactic acid bacteria.

Hydrophobicity

Determining of bacterial adherence to hydrocarbons was performed as described by Rosenberg (26) which is generally known as MATH or BATH test. *Strep. thermophilus* ST8.01 was showed 67.23±7.16% affinity to xylene. Previous studies indicated that lactic acid starter species have lower hydrophobicity (under 32%) than other lactic acid bacteria. However most of the probiotic bacteria among the lactic starters have higher affinity to hydrocarbons than 32% (18). Although studies for determining of hydrophobicity of *Strep. thermophilus* have been rarely done and it is reported that hydrophobicity of *Strep. thermophilus* varied from 24% to 98% depending on their source (48). In this study hydrophobicity value of *Strep. thermophilus* ST8.01 was also found higher than many of the *Strep. thermophilus* strains (67.23±7.16%) as similar with the study of Iyer et al. (29).

Antibiotic Susceptibility

Strep. thermophilus ST8.01 was assayed to 10 antibiotics using disc diffusion method. Strain ST8.01 exhibited complete susceptibility to nine antibiotics. This strain showed moderate susceptibility to only sulphamethoxazole/ trimethoprim. Previous studies confirmed that *Strep. thermophilus* is usually showed susceptibility to tetracycline, erythromycin, chloramphenicol, cephalothin, quinupristin/dalfopristin and ciprofloxacin while it has moderate susceptibility to high resistance to gentamicin, kanamycin and streptomycin (49-51). It is also reported that *Strep. thermophilus* used in yoghurt production has not been resistant to ampicillin and penicilin (31, 52), as strain ST8.01. Tosi et al. (53) determined that *Strep. thermophilus* strains can show atypic antibiotic resistance patterns. In their study some strains were shown resistant to tetracycline and clindamycin while some other resistant to

tetracycline and sensitive to erythromycin at the same time. The major problem is considered as the antibiotic genes might be transferred to pathogenic bacteria including *Streptococci, Listeria* and *Enterococci* in the gastrointestinal tract or in digested foods. For this reason complete susceptibility to clinically important antibiotics of *Strep. thermophilus* ST8.01 is adventageous.

CONCLUSION

In this study, maintenance the vitality of *Strep. thermophilus* ST8.01 under some gastrointestinal stress conditions (bile salt, lysozyme, phenol and situmulated gastric juice); having autoaggregation and hydrophobicity abilities are required properties for probiotic cultures. Besides these features, if we consider rapid acidification, good proteolitic activity and good flavour compound production of EPS-producing *Strep. thermophilus* ST8.01 (20), this strain may be a candidate for probiotic starter culture.

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