

Antioxidant and Antibacterial Activities of Exopolysaccharides Produced by *Lactic Acid Bacteria* Isolated from Yogurt

ZOUAOU BENATTOUCHE^{a*}, DJILLALI BOUHADI^b, AND GHALEM BACHIR RAHO^c

^a Laboratory of Bioconversion, Microbiology Engineering and Alimentary Security, Department of Biology Mascara University - 22900 Algeria

^b Laboratory of Bioconversion, Microbiology Engineering and Alimentary Security, Department of Biology Mascara University- 22900 Algeria

^c Laboratoire de Microbiologie Moléculaire Proteomics et Santé, Department of Biology Sidi Belabbes University- 22300 Algeria

*Corresponding author

benattouche_22@yahoo.fr

TEL: +213557172691

FAX: +21349646312

Received: 19 July 2017; Published online: 18 October 2018

Abstract

The objectives of this study were to optimize the conditions for cell growth and exopolysaccharides (EPS) production by using pure and mixed microbial cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, and to evaluate the antioxidant and antibacterial activities of EPS *in vitro*. The mixed cultures of two strains showed a higher cell growth whereas the higher EPS production was detected in pure culture with *S. thermophilus*. The optimal medium were determined as follows (g/l): sucrose 50, yeast extract 10, KH₂PO₄ 3, MgSO₄·7H₂O 0.05 and pH initial 6.5 at 30 °C. Under the optimized conditions, the values of dry cell weight (DCW) and EPS were 5.2 ± 0.8 g/l and 56.8 ± 0.62 mg/ml, respectively. The EPS demonstrated a positive antioxidant potential on DDPH radical scavenging. The agar diffusion assay showed that purified EPS exhibited antibacterial activities against tested pathogens such as *Escherichia coli* ATCC 250922 and *Staphylococcus aureus* ATCC 250923 at (62-1000) µg/mL. In conclusion, EPS have an antioxidant activity and could have applications in the food industry.

Keywords: Xopolysaccharide; antioxidant and antibacterial activities; *Streptococcus thermophilus*; *Lactobacillus bulgaricus*

1 Introduction

Recently, lactic acid bacteria (LAB) have received attention for their exopolysaccharides (EPS) producing ability. A broad range of EPS from LAB with variable functionality can have a wide range of industrial application (Cerning & Marshall, 1999) as gelling agents, biosurfactants, emulsifiers, viscosifiers (Poli, Anzelmo, & Nicolaus, 2010) and biosorbents (de Oliveira Martins, de Almeida, & Ferreira Leite, 2008) In addition,

EPS play important roles in human health owing to their antitumor, antioxidant, anti-ulcer and antibacterial activities (Kocharin, Rachathewee, Sanglier, & Prathumpai, 2010; Liu, Chu, Chou, & Yu, 2011) However, owing to the lack of expansive knowledge on EPS from food-associated LAB, EPS have remained largely underexploited. Fermentation is a very versatile process technology for producing value added products such as microbial biopolymers and since fermentation

parameters have a high impact upon the viability and economics of the bioprocess, their optimization holds great importance for process development. Especially, microbial polysaccharide production is greatly influenced by fermentation conditions such as pH, temperature, oxygen concentration and agitation as well as by the composition of the culture medium (Bryan, Linhardt, & Daniels, 1986).

Yogurt is a fermented dairy product resulting from the symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* during milk fermentation. A classical yogurt starter culture produces a smooth viscous gel, with a desirable fermented product aroma and flavor polysaccharides derived from *S. thermophilus* and *L. bulgaricus* show large variation in composition, charge, spatial arrangement, rigidity and ability to interact with proteins. Furthermore no defining correlation between EPS concentration and viscosity has been established in studies to date (Welman & Maddox, 2003). With the aim of increasing knowledge of the functional EPS from LAB in yogurt, we have isolated LAB from yogurt. Among the LAB, a crude EPS of *S. thermophilus* was obtained, optimized and examined for its antioxidant and antibacterial activities to find a functionally active EPS.

2 Materials and Methods

2.1 Microorganisms

Standard strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for determination of the antibacterial activity. Lactic acid bacteria were isolated from yogurt samples obtained within the Mascara regions of Algeria in 2017.

2.2 Isolation of Lactic Acid Bacterial Strains with High EPS Production

Three EPS producing strains were isolated by selective plating (from yogurt) on MRS Agar and anaerobic incubation at 37 °C for 48h. EPS producing colonies were evaluated by touching

colonies with a sterile inoculating loop. The presence of ropy strands between the loop and the colony as the loop was slowly raised was considered EPS positive. The colonies were picked up and propagated in MRS broth for 24 h. The EPS producing isolates were verified for gram positive and catalase negative rods and cocci. The purity of cultures were verified by spreading cultures on MRS agar, and repeating several times until a pure culture was obtained.

The two EPS producing isolates were revived by transferring 0.5 ml of each frozen stock to 10 ml of MRS broth and incubating at 37 °C for 48h. The revived cultures were reactivated again by transferring 1.0 ml to 10 ml MRS broth and incubating at 37 °C for 48 h. The reactivated cultures were used as standard inoculums culture. In this experiment, the highest EPS producing isolates (*S. thermophilus* and *L. bulgaricus*), were used for EPS production in MRS broth. MRS was used as the EPS production media.

2.3 Growth and EPS Production

Lactic acid bacteria candidates (*S. thermophilus* and *L. bulgaricus*) were isolated from yogurt and used after selection and identification according to Bergey's Manual of determinative bacteriology, 9th edition (Holt, Krieg, Sneath, Staley, & Williams, 1994) and confirmation of identification by the analytical profile index (API) system. Pure and mixed cultures of the strains were tested for cell growth and production of exopolysaccharides. The pure and mixed microbial cultures were inoculated with a ratio of 1% (v/v) into 100 ml MRS broth in 500 ml conical flasks in triplicate. The pH was adjusted to 6.5. The flasks were incubated on a rotary shaker at room temperature for 72 hrs. The exopolysaccharides were precipitated by adding 3 volumes of cold absolute ethanol and stored overnight at 4°C. Finally, the recovered precipitates were redissolved with distilled water and dialyzed against the same solution for 24h at 4 °C (Garcia-Garibay & Marshall, 1991). The total amount of carbohydrates in the polysaccharides was determined by the phenol and sulfuric acid method described by (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The exopolysaccharides production was

expressed as mg/ml and biomass dry weight (g/l) was monitored by measuring the D600nm of the culture in the final fermented samples.

2.4 Optimization of exopolysaccharide production

To study the effect of different parameters, 1% inoculum containing 3.10^8 UFC/ml were inoculated in 100 ml of production medium. EPS production was optimized under different environmental and nutritional conditions viz incubation period (1 to 3 days), carbon sources (glucose, sucrose), nitrogen sources (peptone, yeast extract) and different sucrose concentrations (1, 2, 3, 4, 5 and 6 %)

2.5 Measurement of growth

The optical density at 620 nm was used to monitor cell growth after appropriate dilution of samples (Adebayo-Tayo & A. Onilude, 2008). Growth was measured as the dry weight per volume by centrifugation (5000 g for 10 min) and then dried to a constant weight in an oven at 60 °C overnight to obtain cell dry weight (CDW).

2.6 Determination of total carbohydrate content

Total carbohydrate content was determined by the method of (Dubois et al., 1956). To the dried pellet, 1 mL of 5% phenol and 5 mL of 96% concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 20 min. The optical density of the sample was read spectrophotometrically at 490 nm and total carbohydrate content was calculated, using glucose as the standard.

2.7 Antibacterial activity of exopolysaccharides

The disk diffusion method was used to determine antibacterial activity by quantifying the clear zone of inhibition around the filter paper disk. In this method, the autoclaved filter paper discs were impregnated with 50 μ l of the EPS and

positioned on a nutrient agar plate, seeded with the test organisms, namely *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Penicillin, Gentamicin and Colistin were used as a positive control, while distilled water was used as a negative control. After firm placement of the discs, the plates were incubated at 37 °C in an inverted position for 1-2 days to allow different species of bacteria to grow. The zones of inhibition in discs were measured by a millimeter scale (Bauer, Kirby, Sherris, & Turck, 1966).

2.8 Free Radical Scavenging Activity

The antioxidant activity was determined by the DPPH scavenging assay (Khalaf, Shakya, Al-Othman, El-Agbar, & Farah, 2008). Various concentrations (62.5, 125, 250, 500 and 1000 μ g/mL) of EPS were prepared in separate tubes. Ascorbic acid was used as a reference compound (0.3125, 0.625, 1.25, 2.5 and 5.0 mg/mL). A freshly prepared solution of 0.002 % DPPH (2, 2, Diphenyl-2-Picryl hydrazyl) in methanol was added to each tube containing different concentrations of extracts (2.0 mL). The samples were incubated in the dark at 37 °C for 20 min and read at 515 nm. The data were expressed as the percent decrease in the absorbance compared to the control. The percentage inhibition of radical scavenging activity was calculated.

2.9 Statistical analysis

The experiments were carried out in triplicate and results are given as the mean \pm standard deviation. The data in all the experiments were analysed (Microsoft Excel 2007) for statistical significance using the Student's Test, and differences were considered significant at $p < 0.05$.

3 Results and Discussion

3.1 Isolation of Lactic Acid Bacterial Strains with High EPS Production

In this study, fifty strains of LAB were isolated from yogurt for determination of EPS production. The primary characterization of bacterial isolates indicated that they were gram positive, rods and cocci, catalase negative and anaerobic. All isolates were screened for EPS production in MRS broth, under anaerobic conditions at 37 °C for 48 h as the EPS production period. According to their efficiency for EPS production, all but two isolates were in two categories namely high and weak, with respective EPS concentrations from 1 to 10 g/l and 0.1 to 1 g/l. 30 and 18 of these isolates were in the respective weak and high categories. Two out of the bacterial isolates from two different yogurts EPS (from 10 to over 25 g/l). These two isolates were subjected to complete identification using the API 20 System. They were identified as lactic acid bacterial strains *L. bulgaricus* and *S. thermophilus*.

3.2 Growth and EPS Production Kinetics

Growth Intensity

Dry cell weight (DCW) of the pure bacterial isolates of *L. bulgaricus*, *S. thermophilus* and mixed cultures in MRS broth for 72 h are presented in Figure 1. During the first 20 h of the incubation period growth intensity in MRS medium increased for all three tested cultures. It was observed that the highest intensity (DCW = 10.8 ± 0.3 g/l) was recorded after 48 h by mixed cultures of *L. bulgaricus* and *S. thermophilus* followed by pure cultures of *L. bulgaricus* (DCW = 5.4 ± 0.42 g/l) and pure cultures of *S. thermophilus* (DCW = 4.6 ± 0.8 g/l). After 48 to 72 h of incubation period the growth intensity in MRS medium decreased for all tested cultures. In addition, during 20 - 48 h of the incubation period there was a symmetric growth intensity for the strains grown in MRS broth.

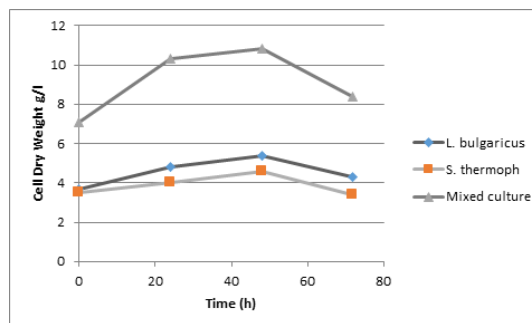


Figure 1: Cell dry weight of isolates of three cultures

EPS production

EPS production from the pure bacterial isolates of *L. bulgaricus*, *S. thermophilus* and mixed cultures grown in MRS broth at 37 °C for 72 h are presented in Figure 2. The highest EPS production and EPS yield % were achieved at 48 hours of growth. The pure cultures of *S. thermophilus* gave the highest EPS concentrations (8.54 ± 0.22 g/l), followed by mixed cultures, and then the pure cultures of *L. bulgaricus*. The pure cultures of *S. thermophilus* were isolated, maintained on nutrient agar, and used throughout the study.

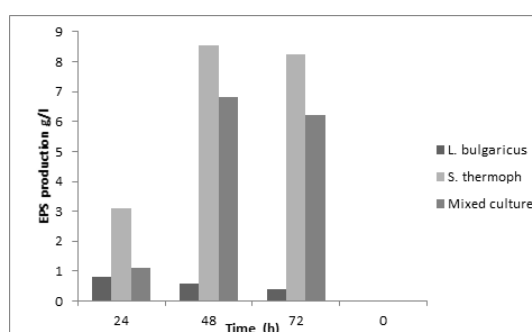


Figure 2: Exopolysaccharide content obtained from isolates of three cultures

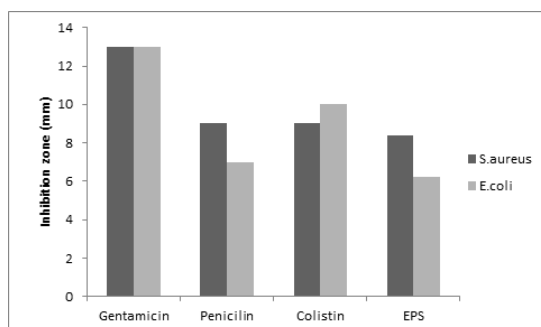


Figure 3: Agar-well diffusion test results of EPS and antibiotics

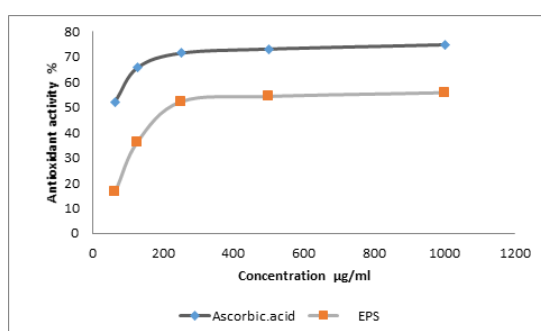


Figure 4: Antioxidant activity of different concentrations of EPS and ascorbic acid in DPPH radical scavenging method

3.3 Optimization of exopolysaccharides

The exopolysaccharide production of *S. thermophilus* was optimized under different environmental and nutritional conditions (Table 1). EPS production was determined during different periods of incubation (1, 2, and 3 days), and was highest after 2 days (8.54 ± 0.22 g EPS/L). Carbohydrates are the most abundant biomolecule and an important nutrient for cell growth and development (Zhang, Zhang, Li, Zhang, & Yang, 2011). The effects of different carbon sources on the production of EPS were studied. In this study, it was found that sucrose was a suitable carbon source (Table 1). However, Antonio Mata et al. (2006) reported that glucose was a suitable carbon source for

EPS production. In this study, a 5% sucrose concentration in the medium gave higher EPS production (56.8 ± 0.62 g/l). Added peptone and yeast extract, as nitrogen sources, had negligible effect on EPS production (56.8 ± 0.62 g/l).

3.4 Antibacterial Activity

Figure 3 shows the antimicrobial activity of purified EPS as evaluated by the disc diffusion method. Purified EPS inhibited the growth of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Antimicrobial activity was maximum when 1000 µg/ml of EPS were used and occurred after 72 h of incubation. The highest and lowest antimicrobial activities of purified EPS were 8.6 - 8.4 mm against *S. aureus* ATCC 25923, and 5.0 - 6.2 mm against *E. coli* ATCC 25922 respectively. Purified polysaccharides had a lower inhibitory effect on growth in comparison with antibiotics frequently used for the treatment of infectious bacteria, such as Gentamicin, Penicillin and Colistin.

The shown sensitivity of gram-positive bacteria to polysaccharides extracts agrees with previous studies (Venturini, Rivera, Gonzalez, & Blanco, 2008). This is due to the membrane composition of the bacterial stains (Holst & Müller-Loennies, 2007). It has been reported that EPS from microorganisms have a strong antimicrobial activity against several pathogens *in vitro*, and several possible antibacterial mechanisms of EPS have been proposed, such as impairment of cell division, disruption of the cell wall and cytoplasmic membrane, and decomposition of DNA (He, Yang, Yang, & Yu, 2010; Wu et al., 2010). According to these results, the antimicrobial activity increased with increasing concentration of EPS.

3.5 Antioxidant activity of EPS

Antioxidant activities have been attributed to various reactions and mechanisms. In this study, the *in vitro* antioxidant activities of EPS from *S. thermophilus* were evaluated using the DPPH radical scavenging assay, and compared with ascorbic acid as a control. The antioxidant activ-

Table 1: Optimization of exopolysaccharide production by *S. thermophilus*

Parameter	Values	EPS g/l	Biomass gr/l
Incubation period (days)	1	3.10 ± 0.3	4.01 ± 0.64
	2	8.54 ± 0.22	4.6 ± 0.8
	3	8.26 ± 0.12	3.4 ± 0.21
Carbon source %			
Glucose	0.5	8.54 ± 0.22	4.6 ± 0.8
Sucrose	0.5	16.02 ± 0.12	4.2 ± 0.24
Sucrose concentration %			
	1	17.4 ± 0.32	4.7 ± 0.62
	2	26.6 ± 0.24	4.8 ± 0.31
	3	33.4 ± 0.46	4.8 ± 0.6
	4	37.2 ± 0.17	4.9 ± 0.2
	5	56.8 ± 0.62	5.2 ± 0.8
	6	51.2 ± 0.6	5.2 ± 0.22
Nitrogen source %			
peptone	0.5	56.8 ± 0.62	5.2 ± 0.5
yeast extract	0.5	56.8 ± 0.62	5.6 ± 0.8

Each value is expressed as mean ±SD

ities of purified EPS at different concentrations (62.5, 125, 250, 500 and 1000 µg/ml) are shown in Figure. 4. The highest antioxidant activity of 55.83 % was found for purified EPS at a concentration of 1000 µg/ml, followed by those at 500 µg/ml (54.34 %), 250 µg/ml (52.24 %), 125 µg/ml (36.12 %), and 62.5 µg/ml (16.34%). In the *in vitro* antioxidant assay, the purified EPS had a DPPH radical-scavenging activity, with an IC₅₀ value of 225 µg/ml which was much higher than that of the standard antioxidant ascorbic acid (48 µg/ml).

The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular weight, structure, conformation, and even the extraction and purification methods. The molecular weight of polysaccharides could play an important role in the antioxidant activity (Chen, Zhang, Qu, & Xie, 2008).

4 Conclusions

In conclusion, the results of this study indicated that the extracellular polysaccharides (EPS) from lactic acid bacteria in yogurt have a significant antioxidant activity in the DPPH system. EPS could be used as antioxidative agents in the food industry.

Acknowledgements

The authors thank the Management of Mascara University for providing laboratory facilities and constant encouragement for this research work.

References

- Adebayo-Tayo, B. & A. Onilude, A. (2008). Screening of lactic acid bacteria strains isolated from some nigerian fermented foods for eps production. 4.

- Antonio Mata, J., Bejar, V., Llamas, I., Arias, S., Bressollier, P., Tallon, R., ... Quesada, E. (2006). Exopolysaccharides produced by the recently described halophilic bacteria *Halomonas ventosae* and *Halomonas anticariensis*. *Research in Microbiology*, *157*(9), 827–835. doi:[10.1016/j.resmic.2006.06.004](https://doi.org/10.1016/j.resmic.2006.06.004)
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, *45*(4), 493–496.
- Bryan, B. A., Linhardt, R. J., & Daniels, L. (1986). Variation in composition and yield of exopolysaccharides produced by *Klebsiella* sp strain-k32 and *Acinetobacter calcoaceticus*-bd4. *Applied and Environmental Microbiology*, *51*(6), 1304–1308.
- Cerning, J. & Marshall, V. M. E. (1999). Exopolysaccharides produced by the dairy lactic acid bacteria. *Recent Results and Developments*, *3*, 195–209.
- Chen, H., Zhang, M., Qu, Z., & Xie, B. (2008). Antioxidant activities of different fractions of polysaccharide conjugates from green tea (*Camellia sinensis*). *Food Chemistry*, *106*(2), 559–563. doi:[10.1016/j.foodchem.2007.06.040](https://doi.org/10.1016/j.foodchem.2007.06.040)
- de Oliveira Martins, P. S., de Almeida, N. F., & Ferreira Leite, S. G. (2008). Application of a bacterial extracellular polymeric substance in heavy metal adsorption in a co-contaminated aqueous system. *Brazilian Journal of Microbiology*, *39*(4), 780–786.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356. doi:[10.1021/ac60111a017](https://doi.org/10.1021/ac60111a017)
- Garcia-Garibay, M. & Marshall, V. M. E. (1991). Polymer production by *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Applied Microbiology*, *70*(4), 325–328.
- He, F., Yang, Y., Yang, G., & Yu, L. (2010). Studies on antibacterial activity and antibacterial mechanism of a novel polysaccharide from *Streptomyces virginia* h03. *Food Control*, *21*(9), 1257–1262. doi:[10.1016/j.foodcont.2010.02.013](https://doi.org/10.1016/j.foodcont.2010.02.013)
- Holst, O. & Müller-Loennies, S. (2007). Microbial polysaccharide structures. In J. P. Kamerling (Ed.), (Chap. Comprehensive glycoscience. From chemistry to systems biology, Vol. 1.04, pp. 123–133). Elsevier.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology*, 9th Ed. Baltimore (Williams & Wilkins, Eds.).
- Khalaf, N. A., Shakya, A. K., Al-Othman, A., El-Agbar, Z., & Farah, H. (2008). Antioxidant activity of some common plants. *Turkish Journal of Biology*, *32*(1), 51–55.
- Kocharin, K., Rachathewee, P., Sanglier, J.-J., & Prathumpai, W. (2010). Exobiopolymer production of *Ophiocordyceps dipterigenabcc* 2073: optimization, production in bioreactor and characterization. *BMC Biotechnology*, *10*(1), 51. doi:[10.1186/1472-6750-10-51](https://doi.org/10.1186/1472-6750-10-51)
- Liu, C.-T., Chu, F.-J., Chou, C.-C., & Yu, R.-C. (2011). Antiproliferative and anticytotoxic effects of cell fractions and exopolysaccharides from *Lactobacillus casei* 01. *Mutation Research-genetic Toxicology and Environmental Mutagenesis*, *721*(2), 157–162. doi:[10.1016/j.mrgentox.2011.01.005](https://doi.org/10.1016/j.mrgentox.2011.01.005)
- Poli, A., Anzelmo, G., & Nicolaus, B. (2010). Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. *Marine Drugs*, *8*(6), 1779–1802. doi:[10.3390/md8061779](https://doi.org/10.3390/md8061779)
- Venturini, M. E., Rivera, C. S., Gonzalez, C., & Blanco, D. (2008). Antimicrobial activity of extracts of edible wild and cultivated mushrooms against foodborne bacterial strains. *Journal of Food Protection*, *71*(8), 1701–1706. doi:[10.4315/0362-028X-71.8.1701](https://doi.org/10.4315/0362-028X-71.8.1701)
- Welman, A. & Maddox, I. (2003). Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends in Biotechnology*, *21*(6), 269–274. doi:[10.1016/S0167-7799\(03\)00107-0](https://doi.org/10.1016/S0167-7799(03)00107-0)
- Wu, M.-H., Pan, T.-M., Wu, Y.-J., Chang, S.-J., Chang, M.-S., & Hu, C.-Y. (2010). Exopolysaccharide activities from probiotic *Bifidobacterium*: immunomodulatory effects (on j774a.1 macrophages) and antimicrobial properties. *International Jour-*

nal of Food Microbiology, 144(1), 104–110.

doi:[10.1016/j.ijfoodmicro.2010.09.003](https://doi.org/10.1016/j.ijfoodmicro.2010.09.003)

Zhang, T., Zhang, C., Li, S., Zhang, Y., & Yang, Z. (2011). Growth and exopolysaccharide production by streptococcus thermophilus st1 in skim milk. *Brazilian Journal of Microbiology*, 42(4), 1470–1478. doi:[10.1590/S1517-83822011000400033](https://doi.org/10.1590/S1517-83822011000400033)