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Influence of "Mild" Sonication Conditions on the Characteristics of *Streptococcus thermophilus* ST-M5

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ABSTRACT

Mild sonication intensity is an acoustic energy which involves the conversion of electrical signal into a physical vibration modifying the permeability of the cell plasma membrane. The objective of this study was to determine the effect of mild sonication intensities at different temperatures on growth, bile tolerance and protease activity of *Streptococcus thermophilus*. The treatments were four mild sonication intensities (8.07, 14.68, 19.83 and 23.55 W/cm^2) randomized at three different temperatures (4˚C, 22˚C and 40˚C). The energy input (1500 J) was kept constant in all treatments. Control samples did not receive any sonication treatment. Growth and bile tolerance were determined every two hours for 12 h of incubation. Protease activity was determined at 0, 12 and 24 h. Mild sonication conditions included a) mild sonication intensities, b) temperatures and c) times, all three of which played a role in influencing the desirable attributes of *Streptococcus salivarius* ssp. *thermophilus* ST-M5. Of all the mild sonication intensities studied, 14.68 W/cm^2 had the best overall influence at certain time points for improving bile tolerance and growth at 4˚C, growth at 22˚C and bile tolerance and growth at 40˚C of *Streptococcus salivarius* ssp. *thermophilus* ST-M5. Mild sonication intensity of 23.55 W/cm^2 had the overall best influence at certain time points for protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 40˚C. *Streptococcus thermophilus* ST-M5 pretreatment with some mild sonication conditions can be recommended for improvement of some of its characteristics.

Keywords: Sonication; Starter Culture; *Streptococcus thermophilus*

1. Introduction

Sonication is an acoustic energy or a sound wave which involves the conversion of an electrical signal into a physical vibration, with a certain frequency and amplitude that can be directed toward a substance [1]. A low sonication condition is a low intensity and non-destructive technique that consists of a series of sound waves with certain frequency, distance, time and temperature [2]. One major consequence of sonication is an event called sonoporation, which is the phenomenon that uses sound for modifying the permeability of cell plasma membranes by applying the acoustic cavitation of microbubbles to enhance delivery. However, it can cause cavitation in aqueous solutions, which is an effective factor in damaging the cell wall of the microorganisms [3]. When a bubble collapses, a high shear rate is generated in the environment that breaks the chemical bonds in the cell wall and membranes [4]. Depending on the strength and frequency of waves, cell wall structure and sonication environment, the impact of ultrasound will be different. It can be classified into two categories based on its outcome: 1) repairable, or reversible, during which the induction of temporary pores on the cell membrane is followed by the pores resealing, leading to cell survival, and 2) lethal, or irreversibly damaged in which the cell is lysed, leading to cell death [5].

Microorganisms (especially spores) are relatively resistant to the effects of sonication, thus extended periods of sonication would be required to render a product safe. Pitt and Ross [6] reported that cells can grow in the presence of low sonication (<2 W/cm^2) due to ability of ultrasound to increase the transport of small molecules (amino acid, peptide, carbon dioxide and water) in solution and the inability of ultrasound to completely remove cells (or even non-living particles) from surfaces. Although the former aspect is well known, the latter is not; in fact its direct opposite is commonly accepted, causing the misconception that ultrasound is efficient in removing cells and particles from surfaces.

Drakopoulou *et al*. [7] found that in the disinfection of wastewater using sonoporation, gram-negative bacteria are more readily susceptible to sonication inactivation than the gram-positive bacteria. Usually, gram-positive organisms have a thicker and a more tightly adherent layer

of peptidoglycans than gram-negative organisms, whereas the latter possesses a lipopolysaccharide that contributes greatly to their structural integrity and protects the membrane from certain kinds of chemical attack. Therefore, the target of ultrasound attack may be the lipopolysaccharide or the inner (cytoplasmic) membrane which consists of a lipoprotein bilayer, since the structure of the peptidoglycan layer does not appear to be a factor [8].

Consumer demand exists for yogurt. Yogurt market was worth $9.5 billion in 2005 and $15.4 billion in 2010 [9]. The percent increase in yogurt sales and consumption between 2008 and 2009 was 5.9% [10]. According to the legal description of yogurt it must contain two microorganisms *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in a 1:1 ratio [11]. The dairy industry has attracted attention to investigating probiotic cultures because of the health benefits associated with their consumption. Among these bacteria, *S. thermophilus* and *L. bulgaricus* are important dairy bacteria from a culture and probiotics standpoint [12]. Upon ingestion of the bacteria they need to survive the bile in the gastrointestinal (GI) tract before they can establish in the lower GI and confer their health benefit on the host, hence bile tolerance is an important characteristic. *Streptococcus thermophilus* possess protease that facilitate casein breakdown to supply amino acids for its growth in milk products. Lactic acid bacteria possess a specific proteolytic activity which degrades the milk proteins to peptides which influence the quality characteristics namely texture and flavor of several aged dairy products such as ripened cheeses. Breakdown of proteins to peptides have been known to make cheeses softer and formation of hydrophobic peptides are known to cause bitterness in cheeses. It is suggested that the proteolytic activity could be a good indicator of showing the ability of probiotic microorganisms to improve the nutritional value of milk products by the formation of bioactive peptides and free amino acids [12]. Probiotic-containing products bring some benefits to consumers and constitute a significant portion of today's emerging functional food sector. They are usually marketed in the form of fermented milk such as yogurt products. In the production of fermented milk products, Kreft and Helen [13]; Wang and Sakakibara [14] have shown that sonication improves the acidifying activity of lactobacilli, thereby reducing production time and while accelerating lactose hydrolysis. It induces a sweetening effect in yogurt without increasing the caloric content.

Energy is the number of joules delivered to the entire batch. This has a close relationship with intensity (W/cm^2), depending on what amplitude is being used [15]. The term amplitude refers to the maximum distance an individual air molecule will move from its starting point as a sound wave passes by Dubbs [4]. The amplitude of a sound wave determines its loudness [4]. A sound wave with large amplitude will sound louder than a small amplitude wave. This is also true for the amount of energy in a sound wave. The intensity increases with increasing amplitude wave [15]. As the percentages of amplitude increase (from 21% to 39%), the sonication time is reduced.

The factors that affect the microbial inactivation with sonication conditions depend on the process (amplitude, time, temperature, and frequency) and microbial entity (type and growth stage of microorganism). Low intensity sonication is a non-destructive technique that consists of the application of low energy, high frequency (1 - 10 MHz) and power intensities below 1 W/cm^2 [16]. McClements [16] reported sonication intensities of up to 1000 W/cm^2 as high intensity sonication. Preliminary work in our lab on screening mild sonication intensities indicated that mild sonication intensities from 8.07 up to 23.55 W/cm^2 had favorable influences on growth of *Streptococcus thermophilus* ST-M5. It is not known if mild sonication intensities between 8.07 and 23.55 W/cm^2 can stimulate yogurt culture bacterium *Streptococcus thermophilus* to enhance its desirable characteristics.

The aim of this study was to elucidate the influence of mild sonication intensities (0, 8.07, 14.68, 19.83 and 23.55 W/cm^2) on the growth, bile tolerance and protease activity of *Streptococcus thermophilus* ST-M5 at refrigeration (4˚C), room (22˚C) and incubation (40˚C) temperatures.

2. Materials and Methods

2.1. Experimental Design

Sterilized peptone water was cooled to 4˚C and individually inoculated with 1% (v/v) *Streptococcus thermophilus* ST-M5 (Chr. Hansen's Laboratory, Milwaukee, WI). The treatments consisted of four mild sonication intensities (8.07, 14.68, 19.83 and 23.55 W/cm^2), randomized at three different temperatures (4˚C, 22˚C and 40˚C) of the peptone water. All treatments had constant frequency (20 kHz) and constant energy (1500 J). The control was the sample that did not receive any sonication treatment at each respective temperature. The control and sonicated samples were tested for growth, bile tolerance, and protease activity. Growth was determined by plating the control and sonicated samples every 2 h for 12 h of incubation. Bile tolerance of the cultures was determined by growing the treated samples in presence of bile and plating every 2 h for 12 h. Protease activity of the control and the sonicated samples was determined by measuring optical density (absorbance units) at 0, 12 and 24 h of incubation of the samples. The experimental design was a completely randomized design (CRD). Three replications were conducted for each experimental condition.

2.2. Sample Preparation

Control and sonicated samples for the growth, bile tolerance, and protease activity analyses were prepared by inoculating 5 mL of freshly thawed pure frozen concentrated stock culture of *Streptococcus thermophilus* ST-M5 (F-DVS, Chr. Hansen's Laboratory, Milwaukee, WI) into 495 mL of sterile 0.1% peptone water at certain temperatures (4˚C, 22˚C, 40˚C) to make it 1% (v/v) and treated in a Sonicator (750 VCX Sonics, Vibracell). *Streptococcus thermophilus* ST-M5 in control and sonicated samples for protease activity were inoculated at 10% (v/v) into sterile skim milk (prior sterilized at 121˚C for 15 min).

2.3. Mild Sonication Treatments

The mild sonication treatment conditions consisted of a constant frequency (20 kHz) and constant energy (1500 J) using four different mild sonication intensities (8.07, 14.68, 19.83 and 23.55 W/cm^2 respectively), randomized at three different temperatures (4˚C, 22˚C and 40˚C) of the peptone water with the culture before it was sonicated.

2.4. Preparation of Media

***Streptococcus thermophilus* Agar (ST Agar)**

ST agar was prepared by mixing 10 g of tryptone, 10 g of sucrose, 5 g of yeast extract and 2 g of dipotassium phosphate (K_2HPO_4) and dissolving in 1 L distilled water. The pH of the solution was adjusted to 6.8 ± 0.1 using 1 *N* HCL, and 6 mL of 0.5% bromocresol purple and 12 g of agar were added to the medium. The medium was boiled and sterilized at 121˚C for 15 min [17].

2.5. Analytical Procedures

2.5.1. Growth

Growth of *Streptococcus thermophilus* ST-M5 was determined by the method proposed by Lin and Young [18] with slight modification. Control and sonicated samples were inoculated (10% [v/v]) into MRS broth (Difco™, Dickinson and Company, Sparks, MD) which was previously autoclaved at 121˚C for 15 min at pH 6.5 ± 0.2. Growth of the culture was determined every 2 h for 12 h of incubation at 37˚C. The inoculated broth (1 mL) was serially diluted in peptone water (0.1% wt/v) and pour plated. The culture *Streptococcus thermophilus* ST-M5 was enumerated using ST agar. During the incubation period for the petri plates of *Streptococcus thermophilus* ST-M5 were incubated aerobically at 37˚C for 24 h [19]. The colonies were then counted.

2.5.2. Bile Tolerance

The bile tolerance was determined according to the method of Pereira and Gibson [20] with slight modifications. The bile tolerance of *Streptococcus thermophilus* ST-M5 was analyzed in MRS-THIO broth [MRS broth (Difco™, Becton, Dickinson and Company, Sparks, MD)] supplemented with 0.3% (wt/v) oxgall (bovine bile) (US Biological, Swampscott, MA) and 0.2% (wt/v) sodium thioglycolate (Acros Organics, Fair Lawn, NJ). Oxgall was added to test bile tolerance of the bacteria and sodium thioglycolate was used in the broth as an oxygen scavenger. Control and sonicated cultures were inoculated at 10% (v/v) separately into MRS-THIO broth and incubated at 37˚C for *Streptococcus thermophilus* for 12 h. The inoculated broth (1 mL) was serially diluted in peptone water (0.1% wt/v) and pour plated every 2 h for 12 h. The cultures *Streptococcus thermophilus* ST-M5 were enumerated using *Streptococcus thermophilus* agar [17]. The petri plates were incubated aerobically at 37˚C for 24 h. The colonies were then counted.

2.5.3. Protease Activity

The extracellular protease activity of *Streptococcus thermophilus* ST-M5 was determined using the *o*-phthaldialdehyde (OPA) spectrophotometric method proposed by Oberg *et al*. [21] with slight modifications.The control and sonicated samples were inoculated (10% [v/v]) into sterile skim milk (prior autoclaved at 121˚C for 15 min), and incubated at 40˚C for 0, 12 and 24 h. After incubation, 2.5 mL from each sample were mixed with 1 mL distilled water and transferred into test tubes containing 5 mL of 0.75 N trichloroacetic acid (TCA) (Fisher Scientific) and the test tubes were immediately vortexed. After holding at room temperature for 10 min, the acidified samples were filtered through a Whatman Number 2 filter paper (Clifton, NJ). Duplicate aliquots from each TCA filtrate were analyzed by OPA using a spectrophotometer (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA). The OPA solution was prepared by combining 25 mL of 100 mM sodium borate (Fisher Scientific), 2.5 mL of 20% (wt/wt) SDS (Fisher Scientific), 40 mg of OPA (Alfa Aesar, Ward Hill, MA) dissolved in 1 mL methanol (Sigma), and 100 µL of β-mercaptoethanol (Sigma) and diluting to a final volume of 50 mL with distilled water. Each TCA filtrate (150 µL) was mixed with 3 mL of OPA reagent in a 3 mL cuvette, and the absorbance at 340 nm was read. Absorbance of the OPA final solution (mixed of OPA reagent with TCA filtrate) with the non-inoculated sterile skim milk (reference sample) was subtracted from each sample reading. OPA final solution was used as a blank to calibrate the spectrophotometer.

2.6. Statistical Analysis

Data were analyzed using Proc Mixed model of Statistical Analysis System (SAS®). Differences of least square means were used to determine significant differences at $P < 0.05$ for main effects (mild sonication intensity, time

and temperature), two way interaction effects (mild sonication intensity × temperature and mild sonication intensity × time) and three way interaction effects (mild sonication intensity × time × temperature). Data are presented as mean ± standard deviation of the means. Significant differences were determined at $\alpha = 0.05$. Significant differences ($P < 0.05$) among the main effects were analyzed using Tukey's adjustment.

3. Results and Discussion

3.1. Growth

The growth of *Streptococcus thermophilus* ST-M5 as influenced by various mild sonication intensities at different temperatures (4˚C, 22˚C and 40˚C) over 12 h are shown in **Figure 1**. There was significant ($P < 0.001$) interaction between mild sonication intensity × time × temperature and between mild sonication intensity × time (**Table 1**). Viable counts increased from 0 to 12 h (**Figure 1**). At 4˚C, 22˚C and 40˚C, all the mild sonication intensities resulted in a significant ($P < 0.05$) increase in viable counts compared to the control from 6 to 12 h of incubation (**Figures 1(a), (b)** and **(c)**).

The mild sonication intensity × temperature was significant ($P < 0.001$) (**Table 1**). Cultures subjected to 8.07, 14.68 and 23.55 W/cm^2 showed better growth at 4˚C than at 22˚C and 40˚C (**Table 2**). The temperature had a significant ($P < 0.001$) effect (**Table 1**). The cultures sonicated at 4˚C showed higher ($P < 0.05$) viable counts compared to the control for 4˚C (**Table 2**).

The mild sonication intensities had a significant ($P < 0.001$) effect (**Table 1**). Some mild sonication conditions increased growth of *Streptococcus thermophilus* ST-M5.

According to Aronsson *et al*. [22], cell physiology could be affected by the application of electrical treatments. In the present study, the application of mild sonication intensities to *Streptococcus thermophilus* ST-M5 at different temperatures enhanced the exponential growth phase after 2 h of incubation. Liong and Shah [23] reported growth of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* to be predominant in the first 9 - 15 h after which it reached a stationary phase. Simova *et al*. [24] analyzed the growth characteristics of *Streptococcus thermophilus* T15 and *L. bulgaricus* HP1 by pre-incubating both cultures for 5.5 h before inoculation and reported that growth reached exponential phase in the first 5 h and stationary phase in 8 - 12 h. Hülsheger *et al.* [25] reported that the application of electric fields to *E. coli* cells increased their survival in the exponential phase of growth. In addition, Kobayashi *et al*. [26] has demonstrated that low intensity of pulsed ultrasound treatments stimulates cell proliferation and production of proteoglycan in human nucleus pulposus cell line, possibly by enhancement of growth factor-related genes. The possible explanation of these results is that the low intensity of pulsed ultrasound vibrates the extracellular matrix and thus subtly alters the environment surrounding the cell thereby stimulating various receptors and adhesion factors on the cell surface [26].

3.2. Bile Tolerance

The bile tolerance of *Streptococcus thermophilus* ST-M5

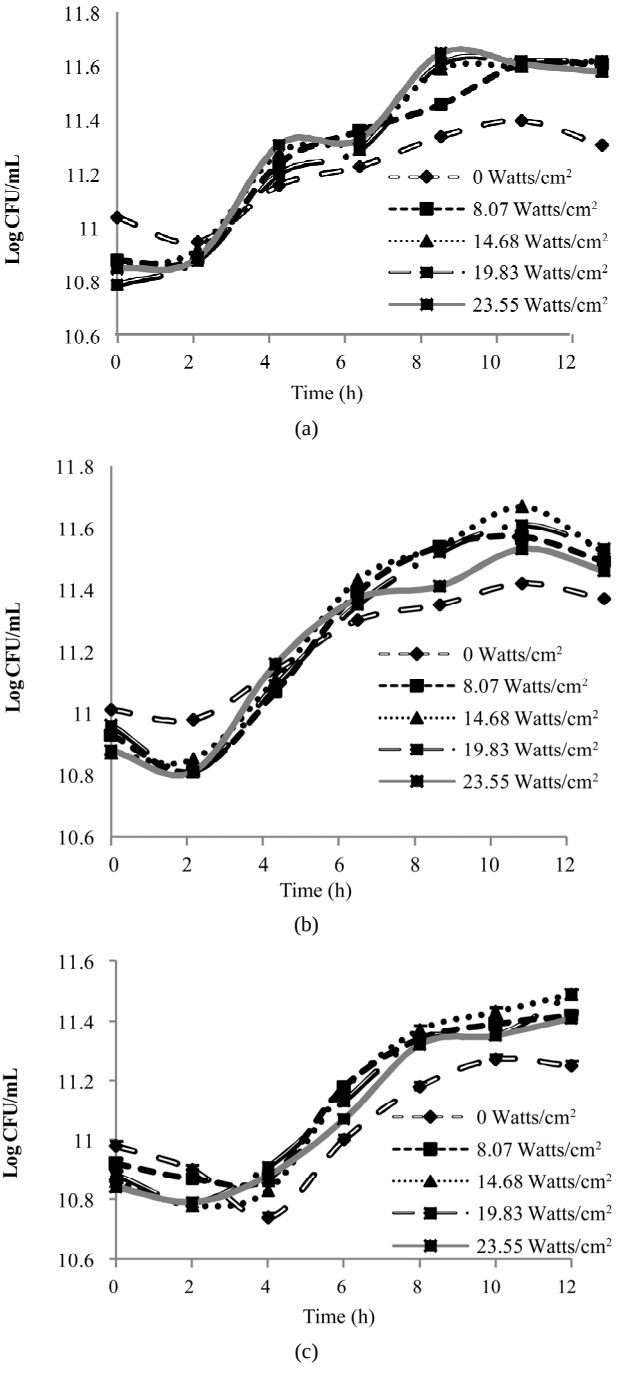

Figure 1. Growth of *Streptococcus thermophilus* ST-M5 at 4˚C (a), 22˚C (b) and 40˚C (c).

Table 1. The Probability > F value of mild sonication intensity, time, temperature and their interactions for acidtolerance, bile tolerance, growth and protease activity of *Streptococcus thermophilus* ST-M5.

	Growth	Bile tolerance	Protease activity[1]
	[3]S.t	S.t	S.t
Effect	Pr > F	Pr > F	Pr > F
INT[2]	<0.0001	<0.0001	<0.0001
TIME	<0.0001	<0.0001	<0.0001
TEMP	<0.0001	<0.0001	<0.0001
INT × TIME	<0.0001	<0.0001	0.5350
INT × TEMP	<0.0001	<0.0001	<0.0001
INT × TIME × TEMP	<0.0001	<0.0001	<0.0001

[1]Time = Incubation period of 24 h for protease activity. [2]INT = Mild sonication intensity. [3]S.t = *Streptococcus thermophilus* ST-M5.

Table 2. The least square means (log_{10}) for growth, bile tolerance, and protease activity of bacteria as influenced by mild sonication intensities.

		Streptococcus thermophilus		
Parameter	Watts/cm^2	4˚C	22˚C	40˚C
		LS mean	LS mean	LS mean
Growth	0	$11.19^{C,b}$	$11.22^{C,a}$	$11.05^{C,c}$
Growth	8.07	$11.28^{B,a}$	$11.25^{B,b}$	$11.14^{A,c}$
Growth	14.68	$11.30^{A,a}$	$11.28^{A,b}$	$11.13^{A,c}$
Growth	19.83	$11.28^{B,a}$	$11.27^{AB,a}$	$11.13^{A,b}$
Growth	23.55	$11.31^{A,a}$	$11.23^{C,b}$	$11.09^{B,c}$
Bile tolerance	0	$8.52^{A,c}$	$10.22^{A,b}$	$10.51^{B,a}$
Bile tolerance	8.07	$8.54^{A,c}$	$10.13^{B,b}$	$10.31^{C,a}$
Bile tolerance	14.68	$8.48^{A,c}$	$10.12^{B,b}$	$10.57^{A,a}$
Bile tolerance	19.83	$8.42^{B,c}$	$10.18^{A,b}$	$10.55^{B,a}$
Bile tolerance	23.55	$8.44^{B,c}$	$10.10^{C,b}$	$10.52^{B,a}$
Protease activity	0	$0.05^{B,b}$	$0.11^{A,a}$	$0.12^{C,a}$
Protease activity	8.07	$0.07^{A,b}$	$0.09^{AB,a}$	$0.09^{D,a}$
Protease activity	14.68	$0.06^{AB,b}$	$0.09^{B,a}$	$0.11^{C,a}$
Protease activity	19.83	$0.07^{A,c}$	$0.10^{A,b}$	$0.13^{B,a}$
Protease activity	23.55	$0.06^{AB,c}$	$0.08^{B,b}$	$0.15^{A,a}$

[ABCD]LS means containing a common letter within the same column for the same parameter are not significantly different. [abc]LS means containing a common letter within the same row are not significantly different.

as influenced by various mild sonication intensities at three different temperatures (4˚C, 22˚C and 40˚C) is shown in **Figure 2**. There was a significant ($P < 0.001$) interaction for mild sonication intensity × time × temperature and for mild sonication intensity × time (**Table 1**). Viable counts decreased from 0 to 12 h (**Figure 2**). At 4˚C, bile tolerance of *Streptococcus thermophilus* ST-M5 subjected to 14.68 W/cm^2 was significantly ($P < 0.05$) higher than the control at 10 h (**Figure 2(a)** and **Table 3**). At 22˚C, the control and cultures subjected to 19.83 W/cm^2 showed significantly ($P < 0.05$) higher bile tolerance compared tothe remaining treatments (**Table 2**). At 40˚C, bile tolerance of cultures subjected to 14.68 and 19.83 W/cm^2 was significantly ($P < 0.05$) higher than the control from 8 to 12 h (**Figure 2(c)** and **Table 3**).

The mild sonication intensity × temperature interaction was also significant ($P < 0.001$) (**Table 1**). The control

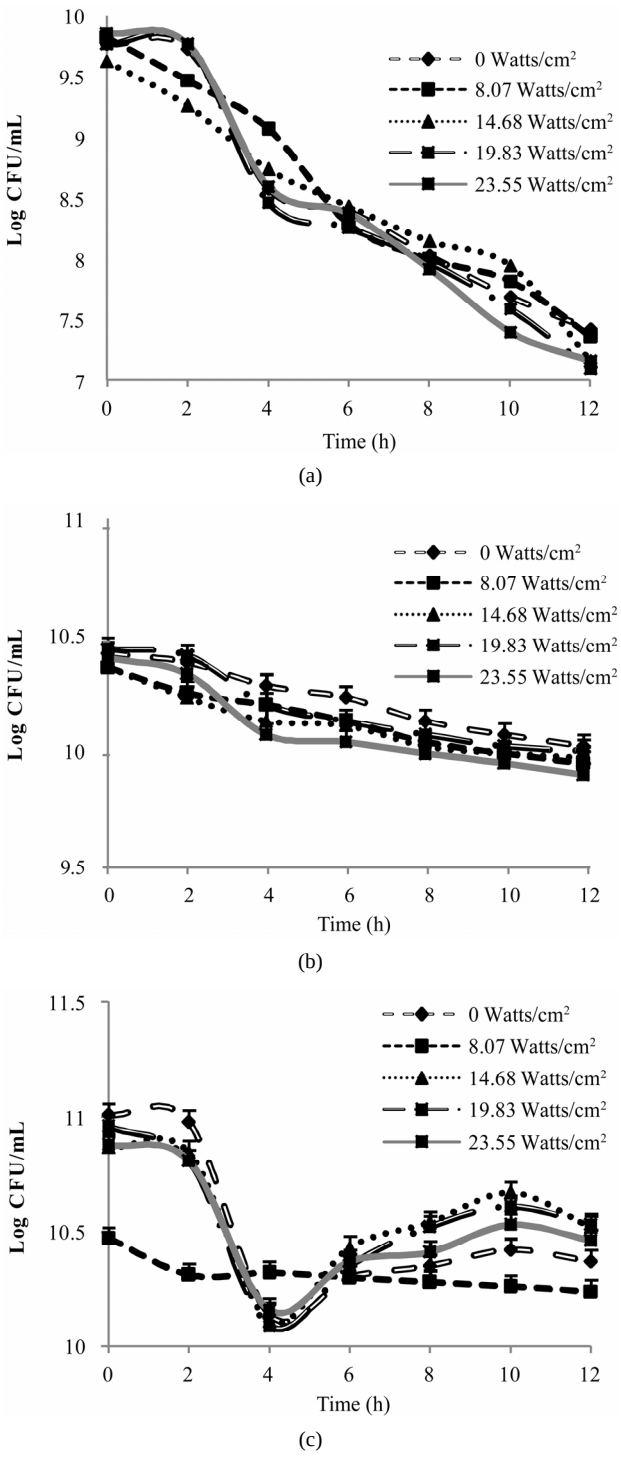

Figure 2. Bile tolerance of *Streptococcus thermophilus* ST-M5 at 4˚C (a), 22˚C (b) and 40˚C (c).

Table 3. The Probability > F value of bile tolerance of *Streptococcus thermophilus* ST-M5 at various mild sonication intensities compared to the control (0 W/cm^2).

Culture	Time (h)	4˚C				22˚C				40˚C			
		Intensity (W/cm^2)				Intensity (W/cm^2)				Intensity (W/cm^2)			
		8.07	14.68	19.83	23.55	8.07	14.68	19.83	23.55	8.07	14.68	19.83	23.55
St[1]	0	0.481	0.044	0.918	0.198	0.381	0.349	0.823	0.762	0.001	0.049	0.491	0.062
St	2	0.001	0.001	0.481	0.481	0.065	0.030	0.615	0.511	0.001	0.048	0.011	0.014
St	4	0.001	0.018	0.132	0.699	0.296	0.038	0.302	0.003	0.009	0.692	0.487	0.734
St	6	0.107	0.581	0.049	0.821	0.148	0.100	0.168	0.009	0.983	0.065	0.417	0.291
St	8	0.764	0.068	0.456	0.112	0.160	0.101	0.393	0.043	0.263	0.006	0.016	0.432
St	10	0.077	0.002	0.148	0.001	0.268	0.307	0.472	0.092	0.022	0.001	0.006	0.100
St	12	0.396	0.001	0.001	0.001	0.343	0.444	0.745	0.097	0.059	0.034	0.021	0.193

[1]*Streptococcus thermophilus* ST-M5.

and all sonication intensities showed better bile tolerance at 40˚C than at 4˚C and 22˚C during 12 h of incubation (**Table 2**). The temperature had a significant ($P < 0.0001$) effect (**Table 1**). *S. salivarius* ssp. *thermophilus* ST-M5 sonicated at 40˚C exhibited the highest bile tolerance at 14.68 W/cm^2 (**Table 2**).

Mean log reduction of the viable counts of *Streptococcus thermophilus* ST-M5 subjected to various intensities at three different temperatures (4˚C, 22˚C and 40˚C) was obtained by subtracting counts at 12 h from 0 h as presented in **Table 4**. In **Table 4**, a high number indicates high bacterial death and a lower number indicates lower bacterial death. At 22˚C, *Streptococcus thermophilus* ST-M5 subjected to 14.68 W/cm^2 had the lowest extent of bacterial death (**Table 4**). At 40˚C, the control (0 W/cm^2) had the highest extent of bacterial death and the mild sonication treatment of 8.07 W/cm^2 showed the lowest extent of bacterial death (**Table 4**). The log reduction using a mild sonication intensity of 8.07 W/cm^2 at 40˚C was 0.23, while the control showed a log reduction of 0.64 indicating that applying 8.07 W/cm^2 increased the rate of survivability of *S. salivarius* ssp. *thermophilus* ST-M5 compared to the control during the incubation (**Table 4**).

Mild sonication intensity had a significant ($P < 0.001$) effect (**Table 1**). Some mild sonication conditions improved bile tolerance of *Streptococcus thermophilus* ST-M5 (**Table 3**). Clark and Martin [27] reported that in the presence or absence of bile acid (Oxgall), *B. longum*and *Lactobacillus bulgaricus*tolerated bile concentrations as high as 4.0%. Furthermore, Shah and Jelen [28] have attributed increased bile tolerance of Lactobacilli strains to their rigid cell wall. There could be other factors responsible for increased bile tolerance of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* when subjected to mild sonication conditions. Bile salts offer antibacterial activity due to the fact that all bacteria have a cell membrane consisting of lipids and fatty acids

Table 4. The mean log reduction of the viable counts of the control and mildly sonicated cultures obtained by subtracting viable log cfu/mL between 0 h and 12 h of incubation in the presence of bile acid (Oxgall) for bile tolerance.

Parameter	Intensity (Watts/cm^2)	*Streptococcus thermophilus* Log cfu/mL		
		4˚C	22˚C	40˚C
Bile tolerance	0	2.35	0.4	0.64
Bile tolerance	8.07	2.46	0.41	0.23
Bile tolerance	14.68	2.47	0.39	0.35
Bile tolerance	19.83	2.67	0.44	0.44
Bile tolerance	23.55	2.70	0.50	0.42

which are susceptible to being dissolved and destroyed by bile salts [29]. Lick *et al*. [30] showed that *Streptococcus thermophilus* and *Lactobacillus bulgaricus* strains are able to survive gastrointestinal passage in vivo and detected viable *S. thermophilus* in human duodenal samples after fresh yogurt ingestion. *Streptococcus thermophilus* showed no significant differences in their growth in MRS broth containing 0%, 0.2% and 0.4% (wt/v) Oxgall for 12 h of incubation at 37˚C when monitored hourly for the growth measured spectrophotometrically at 650 nm [17]. Overall, the data of Begley *et al*. [29] strongly supported the hypothesis that microbial bile salt hydrolase (BSH) functions in the detoxification of bile salts, and in doing so, increases the intestinal survival and persistence of producing strains in the hostile environment of the gastrointestinal tract.

3.3. Protease Activity

The protease activity of *Streptococcus thermophilus* ST-M5 as influenced by various mild sonication intensities at different temperatures (4˚C, 22˚C and 40˚C) is shown in **Figure 3**. There was significant ($P < 0.001$) interaction between mild sonication intensity × time × temperature (**Table 1**). However, the interaction for mild

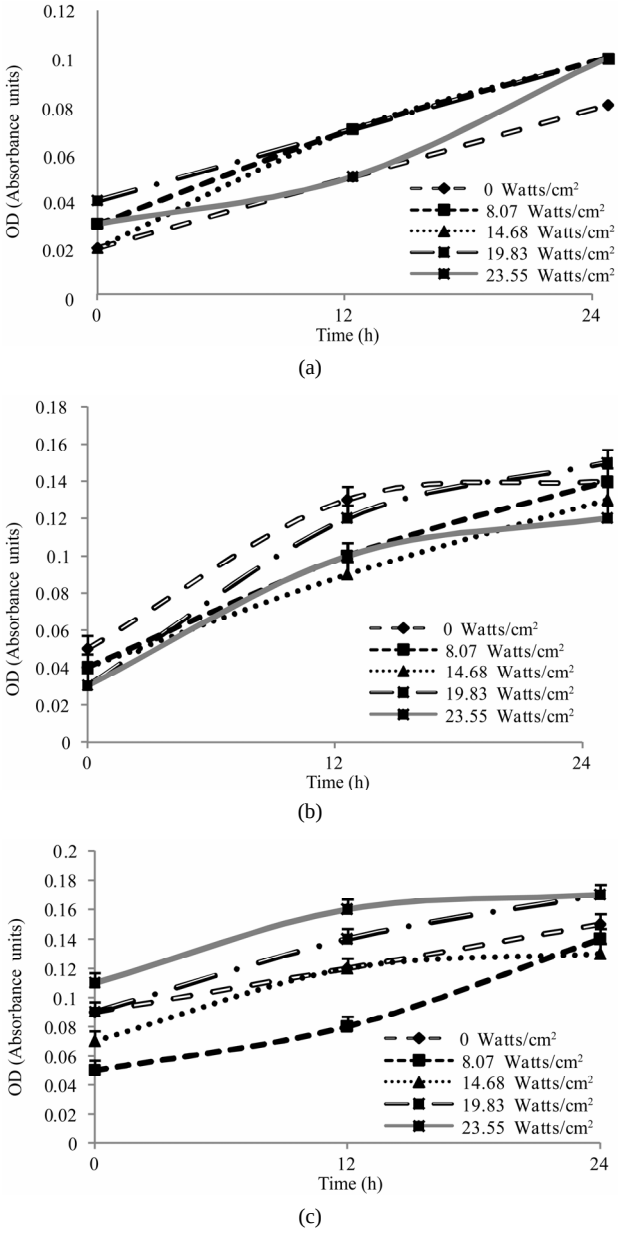

Figure 3. Protease activity of *Streptococcus thermophilus* ST-M5 at 4˚C (a), 22˚C (b) and 40˚C (c).

sonication intensity × time was not significant ($P = 0.535$) (**Table 1**). Absorbance units increased over time from 0 to 24 h (**Figure 3**). At 4˚C, protease activity of cultures subjected to 8.07 W/cm^2 was significantly ($P < 0.05$) higher than the control at 12 and 24 h (**Figure 3(a)** and **Table 5**). At 22˚C (**Figure 3(b)**) the protease activity at all sonication intensities and all time points were lower than the control except for 19.83 W/cm^2 at 24 h. Temperature can denature proteins or influence their action. In the presence of the present sonication conditions at 22˚C the reaction rates appear slowed down hence protease activeity appears to be lower than the control. The significance of this is that if bacterial culture pretreatment for enhanced protease activity (for improved textures and flavors of cultured dairy products) needs to be conducted using these sonication intensities then culture pretreatment at room temperature needs to be avoided. The results are more encouraging at 4˚C. At 40˚C, 23.55 W/cm^2 showed significant ($P < 0.05$) increase in protease activity compared to the control at 0, 12 and 24 h (**Figure 3(c)** and **Table 5**). The optical density (OD) values at 0, 12, and 24 h after using 23.55 W/cm^2 were 0.11, 0.16 and 0.17 absorbance units while OD values for control were 0.09, 0.12 and 0.15 absorbance units at 0, 12, and 24 h, respectively, at 40˚C.

The mild sonication intensity × temperature interaction was significant ($P < 0.001$) (**Table 1**). Cultures subjected to 19.83 and 23.55 W/cm^2 showed better protease activity at 40˚C than at 4 and 22˚C (**Table 2**). The temperature had a significant ($P < 0.001$) effect (**Table 1**). The cultures subjected to 23.55 W/cm^2 at 40˚C showed higher-protease activity than the control (**Table 5**).

The mild sonication intensities had a significant ($P < 0.001$) effect (**Table 1**). Some mild sonication conditions increased protease activity of *Streptococcus thermophilus* ST-M5.

Shah and Jelen [28] reported that *Lactobacillus bulgaricus* exhibited higher *β*-galactosidase activity than *Streptococcus thermophilus* and *Lactobacillus acidophillus*. Additionally, Wang *et al*. [31] sonicated samples of *Lactobacillus delbrueckii* ssp. *bulgaricus* B-5b inoculated in sterile nonfat dried milk for 10 min using a sonicator 300 dismembrator at a frequency of 16 kHz and reported that the highest amount of *β*-galactosidase released by sonication-fermentation was after 4h of the

Table 5. The Probability > F value of protease activity of *Streptococcus thermophilus* ST-M5 at various mild sonication intensities compared to the control (0 W/cm^2).

		4˚C				22˚C				40˚C			
		Intensity (W/cm^2)				Intensity (W/cm^2)				Intensity (W/cm^2)			
Culture	Time (h)	8.07	14.68	19.83	23.55	8.07	14.68	19.8	23.55	8.07	14.68	19.83	23.55
St[1]	0	0.345	0.592	0.117	0.469	0.329	0.132	0.034	0.011	0.001	0.117	0.329	0.049
St12	0.034	0.103	0.140	0.001	0.008	0.001	0.097	0.001	0.001	0.924	0.091	0.001	
St24	0.042	0.037	0.080	0.080	0.776	0.117	0.285	0.075	0.469	0.097	0.057	0.007	

[1]*Streptococcus thermophilus* ST-M5.

culture incubation in milk fermentation. This indicated that the intracellular enzyme was not released into the medium during conventional fermentation, but was released during sonicated fermentation.

4. Conclusion

Mild sonication conditions included a) mild sonication intensities, b) temperatures and c) times, all three of which played a role in influencing the desirable attributes of *Streptococcus thermophilus* ST-M5. Of all the mild sonication intensities studied, 14.68 W/cm^2 had the best overall influence at certain time points for improving bile tolerance and growth at 4˚C, growth at 22˚C and bile tolerance and growth at 40˚C of *Streptococcus thermophilus* ST-M5. Mild sonication intensity of 23.55 W/cm^2 had the overall best influence at certain time points for protease activity of *Streptococcus thermophilus* ST-M5 at 40˚C. *Streptococcus thermophilus* ST-M5 pretreatment with some mild sonication conditions can be recommended for improvement of some of its characteristics.

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