Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium

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ABSTRACT

The aim of this research was to assess the amounts of polysaccharide and surfactin produced by *Bacillus subtilis* ATCC 6633 in rehydrated whey powder (RWP) as the growth medium. One-day-old cultures of *B. subtilis* (~4.6 log cfu/mL) were inoculated into 100 mL of 10, 15, or 20% (wt/vol) RWP and incubated at 30°C for 72 h. To analyze the effects of lactose and protein on polysaccharide and surfactin production, 6 RWP solutions containing different levels of lactose and protein were also used as media. The number of vegetative cells and spores, pH, viscosity, and the concentration of lactose were determined at 0, 24, 48, or 72 h of fermentation. The levels of polysaccharide and surfactin produced after 72 h of fermentation were measured using HPLC and the phenol-sulfuric acid method, respectively. During 72 h of fermentation, *B. subtilis* populations increased from 4.6 to 10.54, 9.82, and 9.67 log₁₀ cfu/mL in 10, 15, and 20% RWP, respectively. The number of *B. subtilis* spores in 10% RWP increased from 3.91 to 4.72 log₁₀ cfu/mL after 48 and 72 h of fermentation, respectively. The increased level of lactose or protein in RWP did not significantly change the vegetative growth. After 72 h of fermentation, the pH of RWP decreased from 5.70 to 4.99 with a slight increase in viscosity. Polysaccharide levels in 10, 15, and 20% RWP after fermentation were 513.6, 613.5, and 768.3 mg/L, respectively, with *B. subtilis* producing 0.18 to 0.29 g/L of surfactin after 72 h of fermentation. The polysaccharide or surfactin production was not changed significantly by addition of protein or lactose to RWP. These results indicate that RWP is a good fermentation substrate for surfactin and polysaccharide production.

Key words: whey powder solution, *Bacillus subtilis*, polysaccharide, surfactin

INTRODUCTION

Extracellular polysaccharides (EPS) are produced by growth of many gram-negative and gram-positive bacteria (Wingender et al., 1999). They are bound to the cell surface and then released into solution. Microbial EPS offer a potential new source of functional biopolymers for food, industrial, and medical applications. These bacterial polysaccharides can be incorporated into food as thickeners, suspending agents, or gelling agents to improve food quality and texture (Stephens, 1995). *Bacillus subtilis* is also a bacterial source for the production of EPS and antibiotics by fermentation (Mitsuda et al., 1981; Desai and Banat, 1997). Some *B. subtilis* strains can produce surfactin, which is one of the most powerful biosurfactants and antimicrobials (Perez et al., 1992; Desai and Banat, 1997; Heerklotz and Seelig, 2001; Stein, 2005).

Whey, a by-product of the dairy industry, has received wide-reaching attention by researchers due to its high nutritional value and functional properties (Horton, 1993). Whey contains lactose (4.9%, wt/vol), protein (0.8%, wt/vol), lipid (0.17%), and trace amounts of vitamins, minerals, and organic acids (Gulsun and Sahan, 1992). It has been investigated as a medium for the growth of various microorganisms (Feijoo et al., 1999; Lee et al., 1997; Yagci et al., 2006). The use of whey as a cheap fermentation substrate for production of polysaccharide, surfactant, enzymes, vitamins, or antibiotics would help to decrease whey disposal. Some studies have showed that whey permeate could be used as a fermentation substrate for *Pseudomonas* spp. or *Klebsiella oxytoca* to produce polysaccharides or rhamnolipid biosurfactant (Stauffer and Leeder, 1978; Dlamini and Peiris, 1997a,b; Rech et al., 1999; Kahyaoğlu and Konar, 2006). However, we found no studies in the literature that investigated the growth of *B. subtilis* in whey or production of polysaccharides and surfactin by whey fermentation.

Consequently, our objectives were (1) to evaluate rehydrated whey powder as a fermentation substrate for the growth of *B. subtilis*, and (2) to assess the production of polysaccharide and surfactin using rehy-

Received January 26, 2012.

Accepted March 4, 2012.

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drated whey powder as the fermentation substrate for *B. subtilis*.

MATERIALS AND METHODS

Culture Preparation

Bacillus subtilis ATCC 6633 was obtained from the Health Department of Refik Saydam Hifzisiha (Ankara, Turkey). The culture was stored in tryptic soy broth (Difco Laboratories, Detroit, MI) containing 6% yeast extract and glycerol at -60°C until use. Two successive transfers (30°C for 24 h) were prepared in 10 mL of tryptic soy broth (Difco Laboratories) using a 1% inoculum, after which the culture was pelleted by centrifugation ($12,000 \times g$ for 30 min at 4°C) and resuspended in sterile water for subsequent inoculation.

Preparation and Inoculation of Rehydrated Whey Powder

Rehydrated whey powder (RWP; 10, 15, and 20% wt/vol) was prepared using whey powder composed of 83 to 85% lactose, 5.5 to 6.5% protein, 5.5% mineral, 2% moisture, and 0% fat obtained from a dairy plant (Milkon, Adapazari, Turkey). After RWP was autoclaved at 121°C for 15 min and cooled to room temperature (24°C), one loop of the *B. subtilis* suspension was added into 100-mL aliquots of 10, 15, and 20% RWP, which were then incubated at 30°C for 72 h. Uninoculated 10, 15, and 20% RWP were prepared as controls.

To determine the effect of protein or lactose concentration in RWP on growth and polysaccharide production of *B. subtilis*, 3 different protein and lactose concentrations of solutions were prepared using RWP. For this objective, RWP solution (20%) was autoclaved for 15 min and centrifuged at $4,500 \times g$ at 4°C for 30 min. Supernatant and yeast extract (Difco Laboratories) were used to prepare solutions containing 2.5, 7.5, and 10.0% lactose and 0.5% protein in sterile distilled water. The pellet and lactose were used to prepare 0.5, 1.0, and 1.5% whey protein solutions containing 2.5% lactose. These solutions were inoculated with one loop of overnight *B. subtilis* culture and incubated for 72 h at 30°C in a shaker incubator.

Bacterial Enumeration and Determination of the Number of Spores

Vegetative cells of *B. subtilis* were quantified in RWP after 24, 48, and 72 h of fermentation at 30°C . Serial dilutions prepared using 0.1% (wt/vol) peptone water were surface-plated on tryptic soy agar (Difco Labo-

ratories) with the plates counted after 25 to 30 h of incubation at 30°C .

Spore populations were determined in RWP after 24, 48, and 72 h of fermentation at 30°C using the rope spore method (Halkman and Akcelik, 2000). Fermented RWP was placed in an 80°C water bath (M96 kpk, Elektro-mag, Mountain View, CA) for 20 min to kill vegetative cells and then cooled in an ice bath. Appropriate serial dilutions in 0.1% peptone water were then surface-plated on dextrose casein-peptone agar (Difco Laboratories), with the plates counted after 48 h of incubation at 37°C .

Determination of Viscosity and pH Measurement

Viscosity of RWP was measured during the 72-h fermentation using a capillary Ostwald viscometer (Schott Geräte GmbH, Mainz, Germany). Fermented RWP (3 mL) or distilled water was placed into the capillary tube of the viscometer and then the tube was placed in a 22°C water bath. After the RWP or distilled water reached line A in the capillary tube, the time for the fluid to flow to line B was recorded using a chronometer; this time interval was then used to calculate the viscosity.

The pH was recorded initially and at 24-h intervals during the 72-h fermentation using a pH meter (pH 211 meter, Hanna, Woonsocket, RI).

Lactose Concentration

The amount of lactose in fermented RWP was determined by infrared spectrometry using a MilkoScan FT-120 (Foss, Rellingen, Germany). Samples were prepared according to manufacturer's recommendation. Samples were diluted 5 times and readings were performed in the mid-infrared range with wavelengths from 3 to $10\text{ }\mu\text{m}$ ($1,000\text{--}5,000\text{ cm}^{-1}$), and the spectra were exported and stored in electronic files. At least 5 independent spectra, each obtained by averaging 3 scans of a sample, were obtained per sample.

Determination of Polysaccharide Concentration

Bacillus subtilis was cultivated aerobically in RWP at 30°C and 150 rpm (orbital shaker). The cells were harvested by centrifugation ($5,000 \times g$, 30 min, 4°C) after which the supernatant solution was then centrifuged ($12,000 \times g$, 30 min, 4°C) to remove residual cells. The EPS was precipitated from the supernatant by adding cold reagent-grade ethanol at a volumetric ratio of 3:1 and stored at 4°C for 24 h (de Brouwer et al., 2002). After centrifugation ($12,000 \times g$, 30 min, 4°C), the pel-

let was dissolved in distilled water and concentrated by ultrafiltration using a 30,000 molecular-weight-cut-off membrane (model 8010, Millipore Corp., Bedford, MA). The retentate was washed twice with deionized water, and finally the polysaccharide was dissolved in deionized water for 24 h, freeze-dried, and stored at -20°C pending analysis. Total carbohydrate levels were estimated using the phenol-sulfuric method (Hanson and Phillips, 1981). A purified form of a gellan gum (Gelzan, Sigma-Aldrich, St. Louis, MO) was used as the standard.

Isolation of and Quantitative Analysis of Surfactin

Crude surfactin was isolated by adding concentrated HCl to RWP after removing the biomass by centrifugation (Cooper et al., 1981). The precipitate that formed at pH 2 was collected, dried, and extracted with dichloromethane. The solvent was removed under reduced pressure to yield an off-white solid. Further purification was achieved by recrystallization. The dichloromethane extract was dissolved in distilled water, adjusted to pH 7 with NaOH, filtered through Whatman no. 4 paper, and then readjusted to pH 2 with concentrated HCl. The white solid was collected as a pellet after centrifugation.

The isolated surfactin pellet was dissolved in 1 mL of methanol followed by charcoal treatment and passed through a 0.22- μm -pore filter. The filtrate was subjected to HPLC on a reversed-phase column (RP-18, 5 μm , 4 $\times$ 250 mm; Merck and Co. Inc., Kelco Division, Sydney, Australia). The column was eluted at a flow rate of 1.0 mL/min with acetonitrile-trifluoroacetic

acid (3.8 mM; 80:20, vol/vol) and monitored at 210 nm. The concentration of surfactin was determined from a calibration curve made with surfactin purchased from Sigma.

Statistical Analysis

All experiments were replicated 3 times. Two-way ANOVA was performed using SAS software (SAS Institute, 1990). Means were compared using the Duncan grouping test at $P = 0.05$.

RESULTS

Bacillus subtilis grew well during the fermentation of RWP. The population of vegetative cells in 10% RWP increased from 4.8 to 8.69 and 10.66 log cfu/mL after 24 and 48 h at 30°C ($P < 0.05$), respectively, and then declined by 0.11 logs after 72 h (Table 1). Similar growth of *B. subtilis* was also observed in 15 and 20% RWP, with numbers increasing 5.4 logs during the first 48 h of incubation and then declining by 0.7 to 0.8 logs. The growth of vegetative cells in nutrient broth only after 48 h of fermentation was significantly more than the growth in the RWP solutions ($P < 0.05$). However, different ratios of lactose and protein in the RWP solutions did not significantly affect vegetative growth ($P < 0.05$).

After 72 h of fermentation, the *B. subtilis* spore count in 10, 15, and 20% RWP was 4.73, 3.83, and 3.69 cfu/mL, respectively (Table 2). The density of spores in nutrient broth was not significantly different from those in RWP solutions ($P < 0.05$). However, fewer than 1.3

Table 1. The density of vegetative cells ($\log_{10}$ cfu/mL) in 10, 15, or 20% rehydrated whey protein (RWP) and RWP containing different ratios of lactose and protein during 72 h of fermentation¹

Medium	Fermentation time (h)			
	0	24	48	72
RWP (% wt/vol)				
10	4.83 $\pm$ 0.4 ^{a,A}	8.69 $\pm$ 0.3 ^{a,B}	10.66 $\pm$ 0.1 ^{a,C}	10.54 $\pm$ 0.8 ^{a,C}
15	4.51 $\pm$ 0.6 ^{a,A}	8.87 $\pm$ 0.2 ^{a,B}	10.24 $\pm$ 0.2 ^{a,C}	9.82 $\pm$ 0.1 ^{a,C}
20	4.67 $\pm$ 0.7 ^{a,A}	8.51 $\pm$ 0.4 ^{a,B}	10.22 $\pm$ 0.7 ^{a,C}	9.67 $\pm$ 0.4 ^{a,C}
Adjusted RWP (lactose/protein; % wt/vol)				
10.0/0.5	4.64 $\pm$ 0.2 ^{a,A}	8.51 $\pm$ 0.5 ^{a,B}	10.19 $\pm$ 0.3 ^{a,C}	9.48 $\pm$ 0.1 ^{a,B,C}
7.5/0.5	4.38 $\pm$ 0.3 ^{a,A}	8.37 $\pm$ 0.7 ^{a,B}	9.44 $\pm$ 0.4 ^{a,B}	9.29 $\pm$ 0.9 ^{a,B}
5.0/0.5	4.49 $\pm$ 0.13 ^{a,A}	8.25 $\pm$ 0.4 ^{a,B}	10.50 $\pm$ 0.2 ^{a,B}	9.90 $\pm$ 0.8 ^{a,B}
2.5/0.5	5.01 $\pm$ 0.5 ^{a,A}	8.52 $\pm$ 0.1 ^{a,B}	10.42 $\pm$ 0.7 ^{a,B}	10.43 $\pm$ 0.6 ^{a,B}
Adjusted RWP (protein/lactose; % wt/vol)				
1.5/2.5	4.18 $\pm$ 0.3 ^{a,A}	8.25 $\pm$ 0.1 ^{a,B}	9.94 $\pm$ 0.1 ^{a,C}	10.33 $\pm$ 0.6 ^{a,C}
1.0/2.5	4.27 $\pm$ 0.6 ^{a,A}	8.41 $\pm$ 0.5 ^{a,B}	10.59 $\pm$ 0.2 ^{a,B}	9.37 $\pm$ 0.7 ^{a,B}
0.5/2.5	4.50 $\pm$ 0.2 ^{a,A}	8.44 $\pm$ 0.6 ^{a,B}	10.07 $\pm$ 0.9 ^{a,C}	8.87 $\pm$ 0.8 ^{a,B}
Nutrient broth	4.45 $\pm$ 0.5 ^{a,A}	9.50 $\pm$ 0.5 ^{b,B}	10.31 $\pm$ 0.8 ^{a,B}	9.92 $\pm$ 0.4 ^{a,B}

^{a,b}Different lowercase letters within a column are significantly different ($P < 0.05$).

^{A-C}Different uppercase letters within a row are significantly different ($P < 0.05$).

¹Values are mean $\pm$ SD ($n = 3$).

Table 2. *Bacillus subtilis* spore populations ($\log_{10}$ cfu/mL) in 10, 15, or 20% rehydrated whey protein (RWP) and RWP containing different ratios of lactose and protein during 72 h of fermentation¹

Medium	Fermentation time (h)			
	0	24	48	72
RWP (% wt/vol)				
10	$<1.3 \pm 0.0^{a,A}$	$2.53 \pm 0.5^{b,B}$	$3.91 \pm 0.2^{b,C}$	$4.73 \pm 0.1^{b,D}$
15	$<1.3 \pm 0.0^{a,A}$	$2.65 \pm 0.3^{b,B}$	$3.17 \pm 0.7^{b,B}$	$3.85 \pm 0.2^{b,B}$
20	$<1.3 \pm 0.0^{a,A}$	$2.31 \pm 0.4^{b,B}$	$3.96 \pm 0.1^{b,C}$	$3.69 \pm 0.1^{b,C}$
Adjusted RWP (lactose/protein; %, wt/vol)				
10.0/0.5	$<1.3 \pm 0.0^{a,A}$	$<1.3 \pm 0.0^{a,A}$	$<1.3 \pm 0.0^{a,A}$	$2.42 \pm 0.0^{a,B}$
7.5/0.5	$<1.3 \pm 0.0^{a,A}$	$2.62 \pm 0.6^{b,B}$	$2.54 \pm 0.4^{b,B}$	$3.82 \pm 0.6^{b,C}$
5.0/0.5	$<1.3 \pm 0.0^{a,A}$	$3.91 \pm 0.1^{c,B}$	$3.16 \pm 0.02^{b,B}$	$3.54 \pm 0.8^{b,B}$
2.5/0.5	$<1.3 \pm 0.0^{a,A}$	$3.47 \pm 0.7^{c,B}$	$3.52 \pm 0.4^{b,B}$	$4.75 \pm 0.7^{b,C}$
Adjusted RWP (protein/lactose; %, wt/vol)				
1.5/2.5	$<1.3 \pm 0.0^{a,A}$	$4.26 \pm 0.1^{d,B}$	$4.52 \pm 0.2^{c,B}$	$4.97 \pm 0.9^{b,B}$
1.0/2.5	$<1.3 \pm 0.0^{a,A}$	$4.03 \pm 0.8^{d,B}$	$4.40 \pm 0.1^{c,B}$	$4.85 \pm 1.2^{b,B}$
0.5/2.5	$<1.3 \pm 0.0^{a,A}$	$4.26 \pm 0.2^{d,B}$	$4.33 \pm 0.6^{c,B}$	$4.84 \pm 0.8^{b,B}$
Nutrient broth	$<1.3 \pm 0.0^{a,A}$	$4.32 \pm 0.1^{d,B}$	$4.99 \pm 0.5^{c,B}$	$4.83 \pm 0.9^{b,B}$

^{a-c}Different lowercase letters within a column are significantly different ($P < 0.05$).

^{A-D}Different uppercase letters within a row are significantly different ($P < 0.05$).

¹Values are mean $\pm$ SD ($n = 3$).

$\log_{10}$ cfu/mL spores were observed during 48 h of fermentation in RWP solution containing 10% lactose and 0.5% protein. The density of spores in the latter RWP solution was significantly less than in the other solutions used ($P < 0.05$).

Prior to inoculation, the pH of 10, 15, and 20% RWP was 5.75, 5.61, and 5.53, respectively (Figure 1a). After 72 h of incubation, the pH of 10, 15, and 20% RWP decreased significantly to 4.99, 4.99, and 5.21, respectively ($P < 0.05$). The significant decrease in pH was parallel to the significant growth of *B. subtilis* and subsequent lactic acid production from lactose. In fact, the correlation between pH and the amount of lactose was 80.9, 99.6, and 97.5% for 10, 15, and 20% RWP, respectively, during 72 h of *B. subtilis* fermentation.

The amount of lactose in 10% RWP significantly decreased from 8.5 to 7.6% after 48 h of *B. subtilis* fermentation ($P < 0.05$; Figure 1b) and then stabilized. In 15% RWP, lactose decreased from 12.75 to 10.95% after 24 h ($P < 0.05$) of fermentation and remained unchanged thereafter. However, in 20% RWP, the level of lactose continued to decrease significantly from 17 to 14.15, 13.6, and 13.05% after 72 h of fermentation ($P < 0.05$).

Viscosity of 10, 15, and 20% RWP increased significantly from 2.1, 2.9, and 3.2 cP to 2.9, 3.5, and 3.9 cP, respectively, after 72 h of fermentation with *B. subtilis* ($P < 0.05$; Figure 1c). After 72 h of fermentation at 30°C with *B. subtilis*, 10, 15, and 20% RWP yielded 513.6, 613.5, and 768.3 mg/L of polysaccharides, respectively, with the increase in polysaccharide parallel to the increase in RWP concentration (Table 3; $P < 0.05$). However, an increase in protein or lactose level in

RWP solution did not significantly change polysaccharide production by *B. subtilis* ($P < 0.05$). The production of polysaccharide by *B. subtilis* in nutrient broth was also not significantly different from that in RWP ($P < 0.05$).

After 72 h of fermentation at 30°C, 10, 15, and 20% RWP yielded, respectively, 0.23, 0.18, and 0.24 g/L of surfactin (Table 3). The amount of surfactin produced by *B. subtilis* in nutrient broth was 0.89 g/L, which was significantly greater than amounts in the RWP solutions ($P < 0.05$). However, increasing the percentage of lactose or protein in RWP did not significantly change surfactin production ($P < 0.05$).

DISCUSSION

Lactose-based media have not been commonly used as fermentation substrates for biopolymer production because most microorganisms, having deficiencies in lactose-hydrolyzing enzymes, cannot utilize lactose (Stauffer and Leeder, 1978; Racine et al., 1991; Flatt et al., 1992; Konicek et al., 1993; Dlamini and Peiris, 1997a,b). *Bacillus subtilis*, which possesses β -galactosidase (a lactose-hydrolyzing enzyme), can utilize lactose as a carbon source, with one study showing that 60 of 130 *B. subtilis* strains produced gluconate from a laboratory medium containing 2% lactose (Stauffer and Leeder, 1978). The increasing vegetative cell populations and decreasing pH values observed in this study support the fermentation of lactose by *B. subtilis*. *Bacillus subtilis* grew faster in 10% RWP than in 15 or 20% RWP during 48 h of incubation at 30°C. The initial amounts of lactose in 10, 15, and 20% RWP

Figure 1. Mean $\pm$ SD (a) lactose concentration (%), (b) pH, and (c) viscosity of *Bacillus subtilis* cultured in 10, 15, or 20% rehydrated whey powder (RWP) during 72 h of fermentation ($n = 3$).

were 8.5, 12.5, and 17.5%, respectively. The presence of a higher lactose concentration (12.5 or 17.5%) in the medium might increase turgor in the cell, and a plateau effect might be reached to regulate the pressure in terms of carbohydrate utilization (Prescott et al., 2002).

Spore formation by *B. subtilis* was also examined in this study due to the close association with production of polysaccharides and antimicrobial agents. Hamon and Lazazzera (2001) found that a mutation in the *spoA* gene of *B. subtilis* that regulates sporulation suppressed production of a 3-dimensional biofilm that was

encased in an EPS matrix. However, no correlation was found between sporulation and polysaccharide production during the 72-h fermentation in the current study. The present study showed that sporulation began 48 h after the start of fermentation. Sporulation was most rapid in 20% RWP that contained 17.5% lactose. However, the density of viable spores in 15 and 20% RWP decreased during the last 24 h of fermentation, likely because of suppression of vegetative growth from increased acidity.

On the understanding that increased lactose utilization will lead to a further increase in polysaccharide production (Schwartz and Bodie 1985), studies have been reported. For example, one strain of *Pseudomonas elodea* exhibited improved lactose utilization after serial transfer in enriched whey broth (24 h) followed by a lactose-rich (4%, wt/vol) medium (4 transfers in 11 d) and whey (2 transfers in 4 d; Dlamini and Peiris, 1997a). The improved strain exhibited 1.6 times as much intracellular β -galactosidase activity compared with the parent. For future studies, the lactose-utilization capacity of *B. subtilis* and polysaccharide production can be improved in the same way.

Colonies on solid media or in highly viscous liquid media produce EPS (Gugliandolo et al., 2003). Viscosity of RWP fermented by *B. subtilis* during 72 h also increased due to EPS production (Mitsuda et al., 1981). One particular polysaccharide produced by *B. subtilis* contains an amino sugar different from D-glucosamine and N-galactosamine, the usual amino sugar components of complex polysaccharides. This compound, which comprises between 1 and 2% of the total polysaccharide or about 0.1 to 0.2% of the bacterial cell dry weight, has been obtained in a pure crystalline state. *Bacillus subtilis* NCIM 2063 was examined by Borgio et al. (2009) for its EPS-producing ability in a basal salts solution, minimal salts medium, nitrogen-free medium, chemically defined medium, milk medium, and sewage from different areas. The highest EPS production (206 mg/L) by *B. subtilis* was observed in nitrogen-free medium after 7 d of incubation at 37°C.

Surfactin has been characterized as a cyclic lipopeptide containing a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and 7 amino acids (Kakinuma et al., 1969a,b,c; Stein, 2005). The peptide is glutamic acid (N-bonded to the carboxylate of the fatty acid)-L-leucine-D-leucine-L-valine-L-aspartic acid-D-leucine-L-leucine (bonded to the 3-hydroxyl function). In the current study, 0.18 to 0.29 g/L of surfactin was produced by *B. subtilis* using lactose and whey proteins as carbon source in RWP solutions. Similarly, when Abushady et al. (2005) used lactose as a carbon source, 0.25 g/L of surfactin was produced by *B. subtilis*. In the current study, surfactin production was increased by

Table 3. Concentrations of polysaccharide and surfactin produced by *Bacillus subtilis* in 10, 15, or 20% rehydrated whey protein (RWP) and RWP containing different ratios of lactose and protein during 72 h of fermentation at 30°C¹

Medium	Polysaccharide (mg/L)	Surfactin (g/L)
RWP (% wt/vol)		
10	513.6 ± 12 ^a	0.23 ± 11 ^a
15	613.5 ± 09 ^a	0.18 ± 10 ^a
20	768.3 ± 15 ^{ab}	0.24 ± 20 ^a
Adjusted RWP (lactose/protein; %, wt/vol)		
10.0/0.5	740.8 ± 24 ^{ab}	0.16 ± 9 ^a
7.5/0.5	749.7 ± 19 ^{ab}	0.30 ± 12 ^a
5.0/0.5	734.9 ± 12 ^{ab}	0.22 ± 8 ^a
2.5/0.5	740.7 ± 20 ^{ab}	0.25 ± 7 ^a
Adjusted RWP (protein/lactose; %, wt/vol)		
1.5/2.5	730.5 ± 11 ^{ab}	0.19 ± 10 ^a
1.0/2.5	723.9 ± 08 ^{ab}	0.20 ± 13 ^a
0.5/2.5	767.2 ± 16 ^{ab}	0.29 ± 18 ^a
Nutrient broth	770.1 ± 14 ^{ab}	0.89 ± 13 ^b

^{a,b}Different lowercase letters within a column are significantly different ($P < 0.05$).

¹Values are mean ± SD ($n = 3$).

using nutrient broth (0.5% peptone, 0.5% beef extract, 0.5% yeast extract) as the medium. However, when *B. subtilis* was grown in a nutrient broth medium for 24 h, surfactin production was only about 0.1 g/l (Arima et al., 1968).

Other studies revealed that antibiotic production by *Bacillus* species is variable, being influenced by changes in pH and the nutritional properties of the medium (Cooper et al., 1981; Chatterjee et al., 1992; Marahiel, 1992; Leifert et al., 1995; Stein, 2005). In this study, the low pH (5.5–5.7) and lack of glucose could have suppressed surfactin production. Abushadyi et al. (2005) reported optimum surfactin production (2.25–3 g/L) by *B. subtilis* at pH 6.5 and 7 in a medium containing 40 g/L glucose 4.5 g/L NH_4NO_3 , 40 mg/L MnSO_4 , and 6 mM FeSO_4 , with surfactin production further improved by the addition of iron or manganese salts.

CONCLUSIONS

In conclusion, *B. subtilis* ATCC 6633 was able to grow in RWP using lactose as a carbon source. At the end of the 3-d fermentation process in RWP, polysaccharides and low amounts of surfactin were produced. Future studies should focus on optimizing the various parameters for surfactin and polysaccharide production by *B. subtilis*, including the pH or nutritional content of the medium.

ACKNOWLEDGMENTS

The authors thank Elliot T. Ryser (Michigan State University, East Lansing) for his critical review of this article. We also thank Milkon Sut Urunleri (Sakarya, Turkey) for providing the whey powder sample. Part of

this work was supported by Sakarya University Scientific Research Projects Fund.

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