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ORIGINAL ARTICLE

Subtilosin A production by *Bacillus subtilis* KATMIRA1933 and colony morphology are influenced by the growth medium

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Abstract *Bacillus subtilis* KATMIRA1933, cultured in modified MRS (de Man, Rogosa, and Sharpe) broth without peptone (animal-free [AF]-MRS), produced subtilosin A at levels similar to, or even higher, than when cultured in MRS broth with peptone. AF-MRS medium contained 2.5 % (w/v) Martone or 2.5 % (w/v) cottonseed hydrolysate instead of peptone and 0.25 % (w/v) bacteriological grade yeast extract instead of normal yeast extract. An increase in cell numbers, accompanied by an increase in subtilosin activity, was recorded when cells were grown in AF-MRS supplemented with 0.4 % (w/v) K_2HPO_4 and 0.02 % (w/v) $MgSO_4$. Subtilosin production increased from 30 arbitrary units (AU) mL^{-1} in a static culture to 320 AU mL^{-1} when cells were agitated on an orbital shaker at 300 rpm. A further increase in subtilosin production, from 150 AU mL^{-1} to 240 AU mL^{-1}, was recorded when cells were cultured in AF-MRS supplemented with 2.0 % (w/v) amylopectin or 2.0 % (w/v) maltodextrin. Slightly higher cell densities were recorded in the presence of maltodextrin. Two colony types, one with a flat ("typical"), sprawling morphology and the other elevated ("atypical"), were isolated from cells grown in AF-MRS broth. Higher subtilosin levels (213 AU mL^{-1}) were recorded from cells grown in AF-MRS broth supplemented with $MgSO_4$ as compared to cells grown in the absence of $MgSO_4$ (150 AU mL^{-1}).

Keywords Subtilosin · *Bacillus subtilis* · Production optimization

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Introduction

Bacteriocins are ribosomally produced proteinaceous substances of bacterial origin that exhibit antimicrobial activity against a wide range of species (Cotter et al. 2005). These proteins are classified based on their possible post-translational modification, mode of action, genetic organization, chemical composition, and molecular size (Riley and Wertz 2002; Cotter et al. 2005). Originally, subtilosin A (also known as subtilosin) was isolated from *Bacillus subtilis* in 1985 (Babasaki et al. 1985). The primary and three-dimensional structure of the bacteriocin was elucidated from multidimensional NMR studies (Kawulka et al. 2004). The *sbo*A gene encodes a 43-amino acid precursor that is post-translationally modified to form a cyclic peptide. The peptide consists of 35 amino acids, covalently attached by amide linkages between an aspargine residue at the N-terminus and a glycine residue at the C-terminus. An atypical inter-residue thioester bridge is formed between the sulfurs of two cysteines and the α-carbon of two phenylalanine residues, while the sulfur of a third cysteine residue is attached to the α-carbon of a threonine residue (Kawulka et al. 2004; Huang et al.

2009). Subtilosin has a net charge of −2 due to the presence of lysine, two aspartates, and one glutamate (Thennarasu et al. 2005). Such an unusual post-translational structure is not typical amongst naturally occurring proteins and groups subtilosin into a separate class of bacteriocins (Kawulka et al. 2003, 2004).

The mode of action of subtilosin was demonstrated in vitro (Thennarasu et al. 2005). Because of its hydrophobic nature, subtilosin A interacts with the hydrophobic core of the phospholipid bilayer in the cell membrane of the target cell. The negatively charged part of the peptide, which is exposed to the environment, interacts with membrane receptors. The tryptophan residue at position 34 is inserted into the cell membrane, which leads to changes in the conformation of the phospholipids, and hence, destabilization. Similar to the bacteriocins subtilin, epidermin, and gallidermin, subtilosin forms pores through specific interactions with the cell membrane (Riley and Wertz 2002; Ogunbanwo et al. 2003a; Bonelli et al. 2006; Sutyak Noll et al. 2011). In vitro studies have shown that, at concentrations much higher than minimal inhibitory concentration (MIC) levels, subtilosin changes to a multimeric form that induces leakage of essential molecules such as small ions and ATP from the target cell. Increased levels of subtilosin had no significant effect on the cell membranes of mammalian cells (Thennarasu et al. 2005; Sutyak Noll et al. 2011).

In a previous study (Sutyak et al. 2008) we reported on subtilosin A production by a food isolate and its antimicrobial activity against several pathogens, including *Listeria monocytogenes, Gardnerella vaginalis*, and *Streptococcus agalactiae* (Abusham et al. 2009; Sutyak Noll et al. 2011). The isolate was originally classified as *B. amyloliquefaciens* and later identified as *B. subtilis* (Karlyshev et al. 2014). Sutyak and co-workers reported on the potential application of subtilosin in feminine personal care products (Sutyak et al. 2008; Sutyak Noll et al. 2011; Turovskiy et al. 2011). Subtilosin may also be used as a food preservative (Cleveland et al. 2001) and in the control of pre- and post-harvested diseases (FDA 2009; Gabriel et al. 2011). The antiviral activity of subtilosin increases its application potential even further (Torres et al. 2013; Quintana et al. 2014).

Little is known about the effect nutrients have on the production of subtilosin. In this study we report on culture conditions and components in growth media that increases subtilosin production.

Materials and methods

Bacterial strains and culture conditions

Bacillus subtilis KATMIRA1933, originally isolated from a fermented dairy beverage (Sutyak et al. 2008), was obtained from the glycerol stock culture collection. The strain was inoculated into a 50-mL Falcon tube with 20 mL animal-free (AF)-MRS broth prepared according to the formulation in Table 1, received from Marcor Development Corporation (Carlstadt, NJ, USA 07072). The Falcon tubes were positioned at an angle of 45° on an orbital shaker (140 rpm) and the cultures were incubated for 8 h at 37 °C. Five-hundred microliters of the culture was then inoculated into a 250-mL glass flask with 50 mL freshly prepared AF-MRS broth and incubated for 15 h at 37 °C (220 rpm).

Micrococcus luteus ATCC 10240 was used as indicator organism. Cells were inoculated into 20 mL TGY broth (30 g L^{-1} Tryptic Soy Broth, Becton, Dickinson and Company, Franklin Lakes, NJ, USA 07417), supplemented with 6.0 g L^{-1} yeast extract (Becton, Dickinson and Company). After 8 h of incubation at 37 °C, 200 μL of the culture was inoculated into 20 mL freshly prepared TGY broth and incubated for a further 8 h on an orbital shaker (140 rpm).

Cell growth and subtilosin production in modified AF-MRS broth

When describing antimicrobial production in various medium conditions (e.g. MRS, AF-MRS), we refer to the activity attributed to subtilosin. According to the recently published draft genome sequence of *B. subtilis* KATMIRA1933 (Karlyshev et al. 2014), only two sets of bacteriocin transport genes were detected, i.e. genes encoding the transport of subtilosin and bacitracin. However, no genes were detected coding for the production of bacitracin or any other bacteriocin (Karlyshev et al. 2014). *Micrococcus luteus* ATCC 10240, one of the reference microorganisms broadly used by the industry and investigators to quantify bacteriocin production, is sensitive to bacitracin. Calculated per producer cell, we observed no significant difference in the arbitrary units of activity between the purified subtilosin and the supernatant samples obtained from growth in MRS or AF-MRS, when checked with *M. luteus* using PAGE overlay method as described by Sutyak et al. (2008). Only the zones of inhibition corresponding to subtilosin (about

Table 1 Composition of AF-MRS

Component	% (w/v)
Dextrose (Difco, Detroit, MI, USA)	2.0
Freetone A-1 (Marcor, Carlstadt, NJ, USA)	1.25
Martone L-1 (Marcor)	1.25
Sodium acetate (Fisher, Fair Lawn, NJ, USA)	0.5
Dibasic ammonium citrate (Fisher)	0.2
K_2HPO_4 (Sigma-Aldrich, St. Louis, MO, USA)	0.2
$MgSO_4$ (Sigma-Aldrich)	0.01
$MnSO_4{\cdot}H_2O$ (Sigma-Aldrich)	0.005
Tween-80 (Sigma-Aldrich)	0.01

3.4 kDa) were observed, and there were no zones of inhibition corresponding to bacitracin (approx. 1.4 kDA) (data not shown). Therefore, we consider our supernatant containing only subtilosin as an antimicrobial agent.

Modifications made to AF-MRS are listed in Table 2. Tween 80 was animal-free grade, obtained from Avantor Performance Materials, Inc. (Center Valley, PA, USA 18034). Concentrations used in the medium varied from none to 0.2 % (w/v). Dextrose was replaced with the same concentration (2.0 %, w/v) sucrose, corn starch, maltodextrin, and amylopectin, respectively. The Freetone A-1 and Martone L-1 (both from Marcor Development Corporation) added ranged from 1.25 % (w/v) to 2.5 % (w/v). In one experiment the Freetone A-1 and Martone L-1 were replaced with 2.5 % (w/v) cottonseed hydrolysate and 2.5 % (w/v) cottonseed hydrolysate-UF (both from Marcor Development Corporation). The yeast extract was either 0.25 % (w/v) or 0.75 % (w/v) bacteriological grade yeast extract, 0.5 % (w/v) processed grade yeast extract (Marcor Development Corporation), or different concentrations (0.25 %, 0.5 % or 0.75 %, w/v) of technical grade yeast extract. Ammonium citrate levels were adjusted to 0.1 % (w/v) and 0.4 % (w/v). Sodium acetate levels were changed to 0.1 % (w/v), 0.2 % (w/v) and 0.4 % (w/v). $MgSO_4 \cdot H_2O$ concentrations were adjusted to 0.0025 % (w/v), 0.005 % (w/v), and 0.02 % (w/v), and $MnSO_4 \cdot H_2O$ to 0.001 % (w/v), 0.0025 % (w/v), and 0.01 % (w/v). K_2HPO_4 levels were changed to 0.1 % (w/v), 0.4 % (w/v), 0.8 % (w/v), 1.6 % (w/v), and 2.0 % (w/v). In all of the experiments, unmodified AF-MRS and MRS served as controls. All experiments were performed in 250-mL glass flasks containing 50 mL medium. The media were autoclaved, cooled to 37 °C, and inoculated with 10 % (v/v) 15-h-old culture of strain KATMIRA1933. All cultures were incubated at 37 °C for 15 h on an orbital shaker (220 rpm). The pH was not controlled. All experiments were conducted in triplicate.

Cell growth was determined by recording optical density (OD) at 600 nm. Dilutions were made with corresponding sterile modified AF-MRS broth, which also served as a blank. After 15 h of growth, 10 mL of the cell-free supernatant (CFS) was collected by centrifugation (30 min, 4500*g*, 4 °C), filter-sterilized through 0.2 μm nitrocellulose membranes (Nalgene, Fisher), and the antimicrobial activity of subtilosin in CFS determined using the well-diffusion method (Cotter et al. 2005). TGY agar plates (1.5 %, w/v, Difco agar) were overlaid with 4 mL soft TGY agar (0.7 %, w/v, agar) containing 10^5 CFU mL^{-1} *M. luteus*. Subtilosin activity was expressed as arbitrary units (AU) per mL culture. Filter-sterilized (0.2 μm nitrocellulose membranes, Nalgene, Fisher) CFS and AF-MRS broth served as controls.

The effect of medium volume and agitation speed on subtilosin production

The effect of medium volume and agitation speed was studied by inoculating triplicate volumes of 25, 50, and 100 mL AF-MRS broth, contained in 250-mL glass flasks, with 10 % (v/v) of a 20-h-old culture of strain KATMIRA1933 (Table 3). The cultures were agitated at 140, 220, and 300 rpm, respectively. One culture of each volume size was not agitated. All cultures were incubated at 37 °C for 15 h. The experiment was repeated with MRS broth, set at an initial pH of 6.6. All experiments were conducted in triplicate. At the end of cultivation, samples of each culture were plated onto MRS agar and incubated at 37 °C for 48 h. Colony morphology was studied with a phase contrast microscope. Cell morphology and motility was studied under a light microscope. Cell growth and the activity of subtilosin were determined as described elsewhere.

Selection of an optimal growth medium

An optimal growth medium (AF-MRS_{OPT}) was composed based on cell growth and subtilosin activity recorded with the different modifications of AF-MRS (Table 2). Two liters of autoclaved AF-MRS_{OPT} broth in a 3-L glass flask was inoculated with an 18-h-old strain of KATMIRA1933. Cultivation was at 37 °C for 15 h on an orbital shaker at preselected agitation speeds. The experiment was repeated with AF-MRS and MRS. Cell growth and the activity of subtilosin were determined as described elsewhere.

Mathematical modeling of growth in modified AF-MRS

Results obtained from growth in modified AF-MRS broth were analyzed using the mathematical model of Klykov et al. (2011), tailored by replacing the parameter "t" (time) with "n" (agitation speed). Two groups of constants were used, i.e. constants for specific growth rates (μ_{max}), growth inhibition (A), and biosynthesis (k^{div}, k^{st}), and constants for biomass (X_{Lim}, X_P, $X_{Lim}{}^{st}$), product (P_{Lim}), and substrate (S_{Lim}). In addition, various types of linear dependents were assumed between the parameters "n" and "t". One dependent corresponded to logarithmic growth (LGP) and the other to decreased growth (GIP). Velocity constants of the model used by Klykov et al. (2011) were determined by time (t). In the present study, velocity constants were determined by changes in agitation speed (n). The constants adopted in our study were directly proportional to the constants used in the model by Klykov et al. (2011), but takes into account the additional coefficients of the linear dependence between time (t) and agitation (n). Coefficients of this linear regression were not established because the dynamics of cell biomass and subtilosin activity in each flask were not determined. In the present study, rate constants were distinguished by

Table 2 Effect of medium components on cell growth and subtilosin production

AF-MRS modification	Zone of growth inhibition (mm)	Standard deviation (σ)	Bacteriocin activity (AU mL^{-1})	Maximal OD_{600} (1:10 dilution)	Initial pH	Final pH
Tween 80						
0	15	1.2	160	0.75	6.1	5.9
0.025 %[a]	15	0.7	160	0.67	6.1	5.9
0.050 %	16	0.5	267	0.65	6.1	5.9
0.100 %	17	1.7	213	0.67	6.1	6.0
0.200 %	16	1.3	267	0.58	6.0	6.0
Control (MRS)[b]	18	1.8	320	0.68	6.4	6.2
Carbohydrates						
None	18	0.3	240	0.47	6.4	7.7
2.0 % dextrose	15	0.3	80	0.58	6.2	5.9
2.0 % sucrose	13	0.5	40	0.62	6.4	5.8
2.0 % corn starch	18	0	160	0.41	6.4	6.7
2.0 % maltodextrin	18	0.4	240	0.56	6.4	6.7
2.0 % amylopectin	18	0.3	240	0.41	6.4	6.7
Control (MRS)	16	0.3	107	0.66	6.3	6.0
Nitrogen						
2.5 % Freetone A-1	17	1.7	133	0.54	6.3	5.8
2.5 % Martone L-1	18	1.4	213	0.60	6.2	5.9
2.5 % Cottonseed Hydrolysate	18	1.3	213	0.65	6.4	6.1
2.5 % Cottonseed Hydrolysate-UF	18	1.4	213	0.76	6.3	6.0
1.25 % Freetone A-1 plus 1.25 % Martone L-1	17	1.6	133	0.59	6.3	5.8
Control (MRS)	19	1.1	267	0.63	6.3	6.0
0 % yeast extract (YE)	16	1.4	144	0.58	6.2	5.8
0.25 % YE (bac grade)[c]	17	1.3	160	0.64	6.2	6.1
0.50 % YE (bac grade)	16	1.1	176	0.58	6.2	6.0
0.75 % YE (bac grade)	17	1.6	213	0.58	6.2	6.1
0.50 % YE (processed)[d]	16	0.5	120	0.49	6.2	5.6
0.25 % YE (tech grade)[e]	17	0.9	224	0.65	6.2	6.0
0.50 % YE (tech grade)	17	1.1	192	0.61	6.2	5.8
0.75 % YE (tech grade)	17	1.2	200	0.61	6.2	6.0
Control (MRS)	17	0.6	160	0.53	6.2	5.9
0 % ammonium citrate	17	1.5	213	0.73	6.2	5.9
0.01 % ammonium citrate	17	2.6	187	0.71	6.0	5.9
0.02 % ammonium citrate	18	2.4	160	0.83	5.8	5.9
0.04 % ammonium citrate	14	2.4	45	0.65	5.6	5.6
Control (MRS)	20	1.7	205	0.46	6.3	5.6
Sodium Acetate						
0 %	18	1.1	373	0.72	5.9	6.3
0.01 %	18	1.2	213	0.62	5.9	6.0
0.02 %	17	1.2	133	0.49	5.9	5.7
0.04 %	14	1.5	93	0.41	6.0	5.8
Control (MRS)	16	0.7	160	0.55	6.3	6.0
$MgSO_4$						
0 %	16	1.7	160	0.47	6.1	5.7
0.0025 %	16	1.6	133	0.51	6.1	5.6
0.005 %	16	1.6	133	0.50	6.1	5.6
0.010 %	16	1.3	120	0.47	6.1	5.7

Table 2 (continued)

AF-MRS modification	Zone of growth inhibition (mm)	Standard deviation (σ)	Bacteriocin activity (AU mL^{-1})	Maximal OD_{600} (1:10 dilution)	Initial pH	Final pH
0.020 %	16	1.3	213	0.51	6.1	5.6
Control (MRS)	16	0.7	160	0.55	6.2	6.0
$MnSO_4$						
0 %	18	1.6	240	0.58	6.1	6.1
0.0010 %	16	2.4	240	0.58	6.1	6.1
0.0025 %	17	2.1	187	0.61	6.1	6.1
0.0050 %	17	2.3	160	0.57	6.1	6.0
0.0100 %	17	2.7	173	0.57	6.1	6.1
Control (MRS)	17	0.5	160	0.58	6.2	5.8
K_2HPO_4						
0 %	0	0	0	0.16	5.6	5.9
0.1 %	18	0.7	140	0.50	5.8	5.8
0.2 %	18	1.6	151	0.63	6.1	5.7
0.4 %	19	1.4	191	0.70	6.3	5.8
0.8 %	18	1.2	160	0.59	6.6	6.0
1.6 %	19	0.5	160	0.55	6.9	6.6
2.0 %	18	1.0	160	0.50	6.9	6.7
Control (MRS)	19	1.9	255	0.68	6.3	5.8

[a] % relates to w/v

[b] Unmodified MRS

[c] bac grade=bacteriological grade (Marcor Development Corporation)

[d] processed=processed grade (Marcor Development Corporation)

[e] tech grade=technical grade (Marcor Development Corporation)

Values listed are the average of three experiments

Table 3 Effect of culture volume and agitation on cell growth and subtilosin production in AF-MRS

Volume (mL)	Agitation (rpm)	Zone of growth inhibition (mm)	Standard deviation (σ)	Bacteriocin activity (AU mL^{-1})	Maximal OD_{600} (1:10 dilution)	Initial pH	Final pH
25	0	11	3.1	87	0.40	6.3	6.0
25	140	16	0.6	133	0.61	6.3	5.9
25	220	18	0.5	267	0.91	6.3	7.0
25	300	17	1.1	320	1.02	6.3	7.2
50	0	11	3.5	73	0.22	6.3	6.0
50	140	14	0.8	187	0.28	6.3	5.6
50	220	16	1.5	187	0.51	6.3	6.0
50	300	16	0.9	187	0.66	6.3	6.7
100	0	8	1.3	30	0.09	6.3	6.0
100	140	13	0.6	60	0.25	6.3	5.6
100	220	14	0.7	133	0.24	6.3	5.5
100	300	15	0.7	133	0.47	6.3	5.6
50[a]	220	17	1.1	267	0.54	6.6	6.3

[a] Unmodified MRS

Values listed are the average of three experiments

parentheses (μ_{max}), (A), (k^{div}), and (k^{st}), which are different from the constants in the model used by Klykov et al. (2011). Parameters determining biomass and subtilosin activity (X_{Lim}, X_P, $X_{Lim}{}^{st}$, and P_{Lim}) do not depend on time and remain constant throughout sampling at different agitation speeds.

Results

Effect of media components on subtilosin production

Cell biomass and subtilosin activity varied with each modification of the growth medium (Table 2). Highest subtilosin activity (267 AU mL^{-1} at OD_{600} 0.58) was recorded in the presence of 0.2 % (w/v) Tween 80. The activity decreased to 213 AU mL^{-1} when cells were grown in the presence of 0.1 % (w/v) Tween 80, despite better growth (OD_{600}=0.67). Subtilosin activity in unmodified MRS broth (control), containing 0.1 % (w/v) Tween 80, was much higher (320 AU mL^{-1}) at more-or-less the same cell density (OD_{600}=0.68) recorded when cells were grown in AF-MRS with the same level Tween 80. Little variation was recorded in the initial and final pH of the cultures (Δ 0.2) during 15 h of cultivation in AF-MRS supplemented with different concentrations of Tween 80 and unmodified MRS.

Subtilosin activity in AF-MRS broth without carbohydrates was much higher (240 AU mL^{-1}) than recorded in the same medium supplemented with dextrose, sucrose, or corn starch (80, 40, and 160 AU mL^{-1}, respectively). High activity (240 AU mL^{-1}) was recorded in AF-MRS supplemented with 2.0 % (w/v) amylopectin. Growth in the presence of 2.0 % (w/v) maltodextrin also yielded an activity of 240 AU mL^{-1}, but this was at higher cell density (OD_{600}=0.56). Lowest activity of subtilosin (40 AU mL^{-1} at OD_{600} 0.62) was recorded when cells were grown in AF-MRS supplemented with 2.0 % (w/v) sucrose. A difference in antimicrobial activity levels was recorded when cells were grown in AF-MRS supplemented with 2.0 % dextrose (80 AU mL^{-1} at OD_{600} 0.58) and unmodified MRS (107 AU mL^{-1} at OD_{600} 0.66) that contained the same concentration of dextrose. The biggest variation in pH, from an initial 6.4 to 7.7 (Δ 1.3), was recorded when cells were grown in the absence of carbohydrates.

Subtilosin activity produced by cells grown in AF-MRS, supplemented with 2.5 % (w/v) Freetone A-1, or a combination of 1.25 % (w/v) Freetone A-1 and 1.25 % (w/v) Martone L-1, was much lower (133 AU mL^{-1} at OD_{600} of approximately 0.55), compared to activity recorded when cells were grown in the presence of 2.5 % (w/v) Martone L-1 (213 AU mL^{-1} at OD_{600} 0.6), or 2.5 % cotton seed (213 AU mL^{-1} at OD_{600} of approximately 0.70). Highest activity (267 AU mL^{-1} at OD_{600} 0.63) was recorded in unmodified MRS. No drastic changes in pH were observed.

Growth in AF-MRS supplemented with 0.25 % (w/v) technical grade yeast extract yielded subtilosin activity of 224 AU mL^{-1} at OD_{600} 0.65. This was higher than that recorded in the presence of 0.75 % (w/v) bacteriological grade yeast extract (213 AU mL^{-1} at OD_{600} 0.58) and 0.75 % (w/v) technical grade yeast extract (200 AU mL^{-1} at OD_{600} 0.61). No drastic changes in pH were observed.

Replacement of organic nitrogen sources (peptone and yeast extract) with ammonium citrate in AF-MRS resulted in an overall lowering of subtilosin activity. Of interest was the increase in subtilosin activity (213 AU mL^{-1} at OD_{600} 0.73) in AF-MRS that did not contain ammonium citrate or any other nitrogen source. No drastic changes in pH were observed when cells were grown in AF-MRS. However, growth in unmodified MRS decreased the pH from 6.3 to 5.6 and increased the activity of subtilosin to 205 AU mL^{-1} (OD_{600}=0.46).

An increase in sodium acetate from 0.1 % to 0.4 % (w/v) in AF-MRS led to a decrease in subtilosin activity from 213 AU mL^{-1} (OD_{600}=0.62) to 93 AU mL^{-1} (OD_{600}=0.41). Highest activity was recorded in AF-MRS with no sodium acetate (373 AU mL^{-1} at OD_{600} 0.72); higher than in unmodified MRS (160 AU mL^{-1} at OD_{600} 0.55). No drastic changes in pH were recorded.

Subtilosin activity increased from 160 AU mL^{-1} (OD_{600}= 0.47) in AF-MRS without $MgSO_4$ to 213 AU mL^{-1} (OD_{600}= 0.51) in the presence of 0.02 % (w/v) $MgSO_4$. $MnSO_4$ had the reverse effect on the activity of subtilosin. Growth in AF-MRS without $MnSO_4$, or in the presence of low concentrations (0.001 %, w/v) yielded an activity of 240 AU mL^{-1} (OD_{600}=0.58), whereas a tenfold higher concentration (0.01 %, w/v) yielded an activity of 173 AU mL^{-1} (OD_{600}= 0.57). The presence of $MgSO_4$ and $MnSO_4$ had no drastic effect on changes in the pH of the culture.

Addition of K_2HPO_4 to AF-MRS led to increased subtilosin activity. However, concentrations of 0.8 % (w/v) K_2HPO_4 and higher led to decreased activity. Highest activity (255 AU mL^{-1} at OD_{600}=0.68) was recorded when cells were grown in unmodified MRS that contained 0.2 % (w/v) K_2HPO_4.

Effect of culture volume and agitation on subtilosin activity in AF-MRS

The effect of medium volume and agitation speed is shown in Table 3. Optimal production of subtilosin (320 AU mL^{-1} at OD_{600} 1.02) was recorded in 25 mL AF-MRS broth, agitated at 300 rpm. No increase in subtilosin activity was recorded when the agitation speed of a 50 mL culture was increased from 140 to 300 rpm (Table 3). However, the activity of subtilosin doubled when the agitation speed of a 100 mL culture in the same flask size was increased from 140 to 220 or 300 rpm (Table 3).

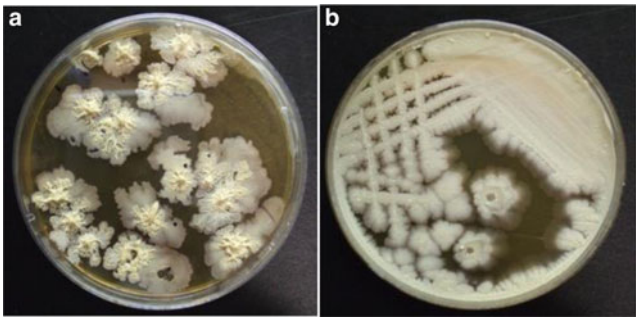

Fig. 1 Colony morphology of variants VP (image **a**) and VF (image **b**) of strain KATMIRA1933 on AF-MRS agar

Plating of the culture produced two different colony types (Fig. 1). The smaller colonies (labeled VF) were cream colored, irregular and nipple-shaped, whereas the larger colonies (VP) were slightly larger, off-white, irregular, and umbonate with lobate margins. Both colony types secreted a mucoid substance. Cells from both colony types were motile rods, but varied in size (2.5 to 4.0 μm in length and 1.0 to 2.0 μm in diameter). Both cell types lost their motility after two days of incubation at 37 °C. The two variants produced subtilosin at more-or-less the same activity levels (213 AU mL^{-1}) in 50 mL AF-MRS broth (Table 4). However, in a mixed culture, activity levels decreased to 150 AU mL^{-1} (Table 4). Increased cell growth of the two variants was observed in MRS, which correlated with an increase in subtilosin activity (Table 4).

Subtilosin activity in an optimal growth medium

The optimal growth medium contained 2.5 % (w/v) Martone or cotton seed hydrolysate, 0.25 % (w/v) yeast extract (technological grade), 0.05 % (w/v) Tween 80, 0.02 % (w/v) $MgSO_4$, and 0.4 % (w/v) K_2HPO_4. The same activity of subtilosin (80 AU mL^{-1}) was recorded after 15 and 26 h of cultivation (Table 5). This corresponded to 60 h of cultivation in unmodified MRS (Table 5). Cultivation in AF-MRS yielded an activity of 20 AU mL^{-1} after 26 h, but 40 AU mL^{-1} over the same period in MRS (Table 5).

Mathematical modeling

The correlation between subtilosin activity and cell density is shown in Fig. 2. The experimental data were analyzed using the Klykov model (2011), as outlined in Table 6, and confirmed the correlation. The main limiting factor that determined subtilosin activity was aeration. Higher levels of subtilosin activity were recorded at higher agitations speeds, or with smaller volumes of culture (Fig. 3). K_2HPO_4 levels higher than 0.1 g L^{-1} had no effect on subtilosin activity (Table 2). Changes in subtilosin activity recorded when cells were grown in the presence of different concentrations of yeast extract and $MnSO_4$ were confirmed with modeling (Tables 2 and 6, respectively). Variations in levels of ammonia, sodium acetate, sugars, peptone, and producer strains do not correspond to changes in subtilosin activity.

Discussion

A number of lipopeptides, such as surfactin, iturin A, fengycin A, and fengycin B, produced by *B. amyloliquefaciens*, inhibit a broad range of plant pathogens and promote the growth of several important crops (Gangadharan et al. 2006; Yusran et al. 2010; Buensanteai et al. 2008). Bacillary-origin α-amylase is widely used in the food industry to improve flour quality and in the starch liquefaction industry to produce sugar and alcohol. Several enzymes produced by *Bacillus* spp. are used in brewing and distilling industries (Reddy et al. 2003; Gangadharan et al. 2006). In addition, α-amylases have been used in clinical, medical, and analytical chemistries and are safe for human consumption (Reddy et al. 2003).

Recently, subtilosin was shown to inhibit *G. vaginalis*, a pathogen causing vaginal infections in millions of women every year (Sutyak Noll et al. 2011). Bacteriocins are naturally occurring bacterial peptides proven to inhibit a wide range of human pathogens while not exhibiting toxicity to human cells (Sutyak et al. 2008; Xie et al. 2009; Tambekar and Bhutada

Table 4 Cell growth and subtilosin production by morphological variants VF and VP of strain KATMIRA1933 in AF-MRS and unmodified MRS

Variant and growth medium	Zone of growth inhibition (mm)	Standard deviation (σ)	Bacteriocin Activity (AU mL^{-1})	Maximal OD_{600} (1:10 dilution)	Initial pH	Final pH
VF, AF-MRS	17	0.5	213	0.48	6.1	5.7
VP, AF-MRS	17	1.9	213	0.52	6.1	5.9
VM[a], AF-MRS	15	0.7	150	0.48	6.1	5.6
VF, MRS (unmodified)	18	0.7	304	0.59	6.1	5.7
VP, MRS (unmodified)	18	2.0	272	0.56	6.1	5.8
VM, MRS (unmodified)	16	1.1	200	0.48	6.2	5.6

VM[a] =combination of VF and VP

All cultures were incubated at 37 °C in 250 mL glass flasks on an orbital shaker (220 rpm) for 15 h. Each flask contained 50 mL medium. Values listed are the averages of three experiments

Table 5 Subtilosin activity in MRS, AF-MRS, and AF-MRS_{OPT} recorded at different time points

Culture medium	Harvest time (h)	Standard deviation (σ)	Bacteriocin activity (AU mL^{-1})	Zone of inhibition (mm)
MRS	15	0	None	None
MRS	26	0.4	40	10
MRS	46	0.4	40	11
MRS	60	0.4	80	13
AF-MRS	26	0.5	20	10
AF-MRS_{OPT}	15	0.7	80	12
AF-MRS_{OPT}	26	0.5	80	13

All cultures were incubated at 37 °C in 3-L glass flasks, placed on magnetic stirrers set at maximum speed. Each flask contained 2 L medium. Values listed are the averages of three experiments

2010; Sutyak Noll et al. 2011). Bacteriocins can be used as an alternative method to prevent pathogenic growth, alone or in combination, with currently existing and/or novel techniques. However, the numbers of currently existing applications are limited, often due to the lack of an appropriate and economically suitable process for bacteriocin production on an industrial scale (this includes cultivation and purification).

Currently, subtilosin can be produced using an expensive animal-based MRS medium. MRS is composed of glucose as a carbon source, two animal-based, laboratory-grade peptones, and a highly processed, expensive yeast extract as nitrogen sources. However, some ingredients of animal origin may be involved in the acquisition of untreatable medical conditions, such as Kreutzfeldt-Jacob Disease (FDA 2009). Therefore, biotechnologically optimized production using plant-derived medium components is absolutely essential for industrial production of substances targeted at human application. Thus, in order to produce subtilosin commercially, cultivation conditions should be optimized. The goal of this study was a preliminary analysis targeted at the development and optimization of an animal-free growth medium for subtilosin production by a) using lower-cost, cultivation-grade ingredients for the nitrogen sources, b) determining the optimal concentrations of each ingredient in the medium, and c) studying other biophysical factors, such as aeration and agitation, hence enhancing bacteriocin production further.

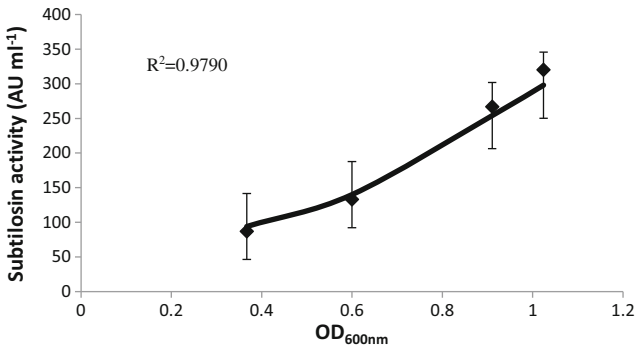

Fig. 2 Correlation between subtilosin activity and cell density of *B. subtilis* KATMIRA1933. Values obtained through experiments were analyzed by using the mathematical model of Klykov et al. (2011). The bacteriocin activity is depicted with diamonds (♦) and two models are shown as (—), GIP model, and (—), LGP model

A classical approach, one variable at a time, was successfully employed to identify essential requirements to scale up subtilosin production. First, medium composition for optimized subtilosin production was formulated using 250-mL shake flasks. Second, physical factors, such as agitation and aeration, optimal for production, were identified. Finally, a bacterial strain producing the highest yield of subtilosin was identified and isolated. As a result, rich and complex MRS medium was replaced by a simple and economically suitable, optimized version (AF-MRS_{OPT}). Animal-derived peptones and meat extract were replaced with equivalent plant-derived ingredients. Use of technical grade yeast extract improved subtilosin production. Tween 80 did not influence either cell growth or subtilosin

Table 6 Klykov's model (2011) parameters for flask incubation at different agitation conditions where "X" is measured in O.D. units

A	X_{Lim}	X_p	μ_{max}	X_l	X_{Lim}^{st}	n_{Lim}	P_{Lim}	k^{div}	k^{st}
0.0125	0.6	1.09	0.0041	0.33	0.30	140	140	5.38	0

LGP is a logarithmic growth phase; ***GIP*** is a growth inhibition phase; *S* substrate concentration, g l^{-1}; *X* is a biomass concentration, *OD* units or g L^{-1}; *OD* is a unit of an optical density; τ is time, hour; *P* is products-metalloproteinase, units *P* mL^{-1}; $A = m/a$ (1) is a parameter that describes a delay of the biomass growth rate; (2) specific rate of accumulation of stable cells, ~h^{-1}; X_p is maximum biomass concentration, when all the energy generated during cultivation is consumed for cell viability maintenance; X_{Lim} is biomass concentration in the end of exponential growth phase and beginning of growth inhibition phase; X^{st} is a concentration of the biomass of zero age cells (stable), the content of resting cells, *OD* units or g L^{-1}; $X^{div} = X - X^{st}$ is a concentration of proliferation biomass, *OD* units or g L^{-1}; τ_{Lim} is a time of exponential growth phase termination, hour; X_{Lim}^{st} is a concentration of stable cells at the end of exponential growth phase, *OD* units or g L^{-1}; n_{Lim} is an agitation at the end of exponential growth phase; *R* is a ratio of X^{st} to biomass *X*, relative content of stable cells in the biomass, synchronization degree, part of 1; k_p^{div}, k_p^{st}, k_s^{div}, k_s^{st} are constants of metabolite and substrate biochemical reaction rates, units of *P* (or *S*)/(mL units of biomass *X* per one hour); X_l is an initial biomass concentration in *LGP* corresponding to the beginning of population structuring, *OD* units or g L^{-1}; P_{Lim} is metabolite concentration or activity at the end of *LGP* and beginning of *GIP*, units of *P* mL^{-1}

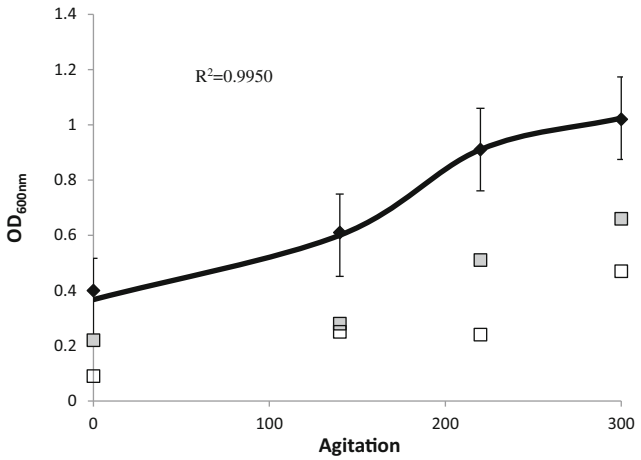

Fig. 3 Effect of agitation and medium volume on growth of *B. subtilis* KATMIRA1933. Values obtained through experiments (plotted as square and diamond symbols) were analyzed using the mathematical model of Klykov et al. (2011), where the 25-mL experiment is depicted with (♦), the 50-mL experiment is shown with (■), and the 100-mL experiment is illustrated with (□). Two models are represented with the lines: (——), 25 mL without limitations, and (——), 25 mL with O_2 limitation as a consequence of the higher cell number

production, which are in agreement with other bacteriocin optimization reports (Trinetta et al. 2008; Han et al. 2011). To decrease the cost and simplify the purification process, Tween 80 concentration was decreased twofold. Dipotassium phosphate appeared to be essential for microbial growth and for bacteriocin production. A number of carbohydrates noticeably improved bacterial growth, but did not influence bacteriocin production. Most likely, the microorganism is capable of producing a sufficient amount of carbohydrates, since it synthesizes α-amylases and other enzymes, which degrade large sugar polymers into monomers (Reddy et al. 2003; Gangadharan et al. 2006; Sutyak et al. 2008). In addition to carbohydrates, compounds inhibiting bacterial growth, such as ammonium citrate and sodium acetate, were also eliminated. Previously conducted studies with *Bacillus* have shown insignificant impact of metal ions on peptide production (Venil and Lakshmanaperumalsamy 2009). However, manganese sulfate was completely removed from the optimized medium since its presence in the medium did not improve bacteriocin production. Scaled up process appeared to be effective in a 7-L bench top bioreactor. In order to improve production, we strongly suggest effective aeration and agitation at 300 to 500 rpm while controlling the foam formation. A number of studies have shown similar effect of aeration and/or agitation on the bacteriocin production by other *Bacillus* spp. (Abusham et al. 2009; Habib et al. 2011). Higher agitation speeds (600 rpm and above) may be employed with antifoam reagents, which had no affect on the cell growth or bacteriocin secreted by *B. amyloliquefaciens*. As a result, an optimized protocol effectively decreased production time from 60 h to only 15 h while allowing for the noticeable improvement of the bacteriocin's yield.

To improve further bacteriocin production, other physical parameters need to be investigated. A number of optimization studies reported a great influence of pH and temperature on the bacteriocin production. In previously conducted studies, bacteriocin activity was shown to be the highest at acidic pH, then decreased and/or became substantially less within basic pH range (Leroy and De Vuyst 1999; Leal-Sanchez et al. 2002; Ogunbanwo et al. 2003b). Cultivation pH often plays an important role in proteolytic activity and may influence cell-to-bacteriocin interactions, thus affecting adsorption of a bacteriocin to the cell wall (Leroy and De Vuyst 1999; Sharma et al. 2010). In order to identify an optimal pH condition for the subtilosin production, further studies should be conducted, perhaps using an indicator organism that is less pH-sensitive than currently used *M. luteus*. In addition, temperature may have a significant impact on the bacteriocin production (Leroy and De Vuyst 1999) and the optimum temperature for the cell growth may not be the same as required for the maximum bacteriocin production (Juarez Tomás et al. 2002). Therefore, a temperature range needs to be studied with a combination of the pH range to identify conditions resulting in maximum bacteriocin activity.

The main limiting factor that determined subtilosin activity was aeration. Higher levels of subtilosin activity were recorded at higher agitations speeds, or with smaller volumes of culture agitated. K_2HPO_4 levels higher than 0.1 g L^{-1} had no effect on subtilosin activity (Fig. 2). The resulting values of bacteriocin correspond well with the model (Table 6), the constants of which correspond with Table 2. This supports that in these concentrations the given salt practically does not affect the productivity of the process, which in this case is also determined by the rate of aeration. The variants with yeast extract and manganese ions (Table 2) also fit the model. Similar to the variation with dipotassium phosphate, there is a predominant influence by oxygen limitation.

In addition to the observed positive effect of aeration on subtilosin production, the following conclusions can be drawn from the study's data: i) elevated concentrations of sodium acetate and manganese sulfate have a negative effect on subtilosin production; and ii) corn starch, maltodextrin, amylopectin, and Tween cause a mild increase in the bacteriocin's concentration observed in cultivations.

To study further the influence of potassium phosphate, magnesium sulfate and the source of nitrogen on subtilosin production, the observed positive effect of aeration should be eliminated or finely tuned. This can be achieved by cultivation in small volumes (e.g. 25 mL and less) at various intensities of agitation (300 rpm and higher) while varying concentrations of the tested substances.

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