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Co-production of surfactin and a novel bacteriocin by *Bacillus subtilis* subsp. *subtilis* H4 isolated from *Bikalga*, an African alkaline *Hibiscus sabdariffa* seed fermented condiment

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ABSTRACT

Bikalga is a *Hibiscus sabdariffa* seed fermented condiment widely consumed in Burkina Faso and neighboring countries. The fermentation is dominated by *Bacillus subtilis* group species. Ten *B. subtilis* subsp. *subtilis* (six isolates) and *Bacillus licheniformis* (four isolates) isolated from traditional *Bikalga* were examined for their antimicrobial activity against a panel of 36 indicator organisms including Gram-positive and Gram-negative bacteria and yeasts. The *Bacillus* spp. isolates showed variable inhibitory abilities depending on the method used. Both Gram-positive and Gram-negative bacteria were inhibited in the agar spot assay while only Gram-positive pathogens were inhibited in the agar well diffusion assay. Cell free supernatants (CFS) of pure cultures of 3 *B. subtilis* subsp. *subtilis* (G2, H4 and F1) strains inhibited growth of *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus* and *Bacillus cereus*, while CFS of 2 *B. licheniformis* (E3 and F9) strains only inhibited *M. luteus*. The antimicrobial substance(s) produced by *B. subtilis* subsp. *subtilis* H4 was further characterized. The antimicrobial substance(s) produced by H4 was detected from mid-exponential growth phase. The activity was sensitive to protease and trypsin, but resistant to the proteolytic action of proteinase K and papain. Treatment with α-amylase and lipase II resulted in a complete loss of antimicrobial effect, indicating that a sugar moiety and lipid moiety are necessary for the activity. Treatment with mercapto-ethanol resulted in a significant loss, indicative of the presence of disulfide bridges. The antimicrobial activity of H4 was heat resistant and active at pH 3–10. PCR detection of *yiwB*, *sboA*, *spoX*, *albA* and *spaS*, *etnS* genes and genes coding for surfactins and plipastatins (fengycins) indicated a potential for subtilosin, subtilin and lipopeptide production, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out and a single band of approximately 4 kDa had antimicrobial activity. Ultra high performance liquid chromatography-time of flight mass spectrometry (UHPLC-TOFMS) analysis of the 4 kDa band allowed identification of surfactin and a protein with a monoisotopic mass of 3346.59 Da, which is dissimilar in size to subtilosin and subtilin. Surfactin is a cyclic lipoheptapeptide, which contains a β-hydroxy fatty acid, but no di-sulfide bridges or sugar residues. The complete loss of activity upon amylase treatment indicates that surfactin was not responsible for the observed antimicrobial effect. However, it cannot completely be ruled out that surfactin acts synergistically with the detected protein, though further investigations are needed to confirm this.

1. Introduction

Bikalga is a fermented food condiment based on *Hibiscus sabdariffa* seeds that serves as a flavoring agent in soups and sauces or as low-cost protein snack in Burkina Faso. It is also known in other African countries under different names such as dawadawa bosto in Niger, datou in Mali, furundu in Sudan and mbuja in Cameroun. The production of *Bikalga* is a traditional process and involves boiling, fermentation, steaming and drying. *Bacillus* spp., especially *Bacillus subtilis* group members have been shown to be the dominant bacteria involved in the fermentation of *H. sabdariffa* seeds into *Bikalga* (Ouoba et al., 2008a). *Bikalga*, like other fermented food condiments forms a

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significant part of the diets of many people in developing countries (Steinkraus, 1996). However, the fermentation step is spontaneous, uncontrolled and usually with varied fermentation times, temperatures and microbiological profiles, resulting in products inconsistent in quality attributes. Occasionally pathogenic bacteria such as *Bacillus cereus*, *Staphylococcus* spp. *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Clostridium* spp. have been found in alkaline fermented foods giving rise to concern about the safety (Parkouda et al., 2009). There is consequently a need to apply modern biotechnological techniques like the use of starter cultures in improving traditional food processing technologies (Achi, 2005). The use of starter cultures with desirable technological properties dominating the fermentation process and preventing the growth of pathogenic and spoilage microorganisms during controlled fermentation of African fermented condiments would lead to products of consistent taste and quality, as well as improved marketability (Holzapfel, 2002; Oguntoyinbo et al., 2007; Ouoba et al., 2008b). Members of the *B. subtilis* group sensu lato include a variety of industrially important species that have a history of safe use in food and industry (Pedersen et al., 2002) and are considered good producers of antimicrobials including bacteriocins, Bacteriocin-like substance (BLS) and lipopeptide antibiotics (Abriouel et al., 2010; Stein, 2005). The proteinaceous natures of bacteriocins imply a putative degradation in the gastrointestinal tract of humans and animals, suggesting their use as natural preservatives in foods (Bizani et al., 2005; Cleveland et al., 2001). Subtilin (Banerjee and Hansen, 1988), subtilosin A (Stein et al., 2004; Sutyak et al., 2008), ericin (Stein et al., 2002) and sublancin (Paik et al., 1998) are bacteriocins produced by *B. subtilis* group species.

Lipopeptides probably represent the most common class of secondary metabolites produced by *Bacillus* spp. (Stachelhaus et al., 2002). They are classified into three different families depending on the amino acid sequence: surfactins/lichenysins, iturins (mycosubtilin, iturin A, and bacillomycin), and fengycins/plipastatins (Ongena and Jacques, 2008). The primary structure and gene organization of the operons encoding non-ribosomal peptide synthetases for the *Bacillus* cyclic lipopeptides surfactin, plipastatin, fengycin, bacillomycin, iturin, and mycosubtilin have been described (Duitman et al., 1999; Galli et al., 1994; Konz et al., 1999; Koumoutsi et al., 2004; Menkhaus et al., 1993; Nakano et al., 1991; Tosato et al., 1997; Tsuge et al., 2001). Recently, based on the alignment of nucleic sequences of adenylation and thiolation domains, Tapi et al. (2010) designed specific degenerated primers, which could detect non-ribosomal peptide synthetase genes particularly involved in lipopeptide biosynthesis in *Bacillus* strains.

The objective of the present study was to screen strains of *B. subtilis* subsp. *subtilis* and *B. licheniformis*, two species that are predominant during *Bikalga* fermentation, for their antimicrobial activity against various pathogenic microorganisms; Further to characterize and identify the antimicrobial compound(s) produced by a selected *B. subtilis* subsp. *subtilis* isolate. In addition the ability of the selected strain to produce antimicrobial substance(s) in *H. sabdariffa* based broth was investigated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Ten *Bacillus* isolates (G2, C3, C6, H4, F1, I7, E3, F9, J3, E5) isolated from different productions of *Bikalga* (Ouoba et al., 2008a) were investigated in the present study. Bacterial strains used as indicator microorganisms included 30 Gram-positive and Gram-negative pathogenic bacteria and 6 pathogenic yeasts obtained from different sources (Table 1). Bacteria were maintained as stock cultures at −80 °C in appropriate broth medium, Brain Heart Infusion (BHI, Oxoid CM1135 Basingstoke, Hampshire, England), or nutrient broth (Oxoid, CM0001)) supplemented with 20% (v/v) glycerol. The yeast were maintained in Yeast Glucose Peptone (YGP) broth [made of 1% (w/v) bactopeptone (211677, Becton, Dickinson, NJ, USA), 1% (w/v) glucose (Merck

Table 1
Indicator strains used in the present study.

Strains	Media*/ temperature (°C)	Origin and/ or reference
Gram-positive		
Bacillus cereus MADM 1291	BHI/37	Birthday cake (food poisoning), Brazil
B. cereus MADM 1561	BHI/37	Cooked chicken (food poisoning), Brazil
B. cereus NVH391-98	BHI/37	Food poisoning, kindly provided by INRA, France[a]
B. cereus 007525	BHI/37	Stew (food poisoning)[b]
B. cereus F4810-72	BHI/37	Emetic food poisoning[c]
B. cereus NC 7401	BHI/37	Emetic food poisoning[d]
B. cereus Ba18H2	BHI/37	Cereulide producer isolated from Sonru[e]
B. cereus F3752A/86	BHI/37	Culture collection of London Metroplitan University
B. cereus LMG13569	BHI/37	Culture collection of London Metroplitan University
Listeria monocytogenes 057	BHI/37	Culture collection of Copenhagen University
L. monocytogenes L028	BHI/37	Culture collection of Copenhagen University
L. monocytogenes Scott A	BHI/37	Culture collection of Copenhagen University
L. monocytogenes NCTC 9863	BHI/37	Culture collection of London Metroplitan University
Micrococcus luteus SKN 624	NA/30	Culture collection of Copenhagen University
M. luteus AT49732	NA/30	Culture collection of London Metroplitan University
Staphylococcus aureus NCTC 10656	BHI/37	Culture collection of London Metroplitan University
Gram-negative		
Salmonella Typhimurium SKN 1155	BHI/37	Animal
S. Typhimurium SKN 533	BHI/37	Culture collection of Copenhagen University
S. Typhimurium O:1036340P/t49	BHI/37	Culture collection of London Metroplitan University
Salmonella Nigeria SKN 1160	BHI/37	Cocoa beans
Salmonella Thompson SKN 565	BHI/37	Culture collection of Copenhagen University
Salmonella Oranienburg SKN 1157	BHI/37	Human
Salmonella Infantis SKN 557	BHI/37	Culture collection of Copenhagen University
Salmonella Enteridis P167807	BHI/37	Culture collection of London Metroplitan University
Yersinia enterocolitica 6A28 SKN 599	BHI/37	Culture collection of Copenhagen University
Y. enterocolitica 8A30 SKN 601	BHI/37	Culture collection of Copenhagen University
Y. enterocolitica BT3ST5,27	BHI/37	Culture collection of London Metroplitan University
Escherichia coli 81 *nr.*149 SKN 541	BHI/37	Culture collection of Copenhagen University
Shigella dysenteria 370	BHI/37	Culture collection of London Metroplitan University
Shigella flexneri USCC 2007	BHI/37	Culture collection of London Metroplitan University
Yeasts		
Candida tropicalis	YGP/30	Isolated from Fura[f]
Candida kefyr	YGP/30	Isolated from Fura[f]
Candida krusei	YGP/30	Isolated from Fura[f]
Candida albicans	YGP/30	Blood sample
Saccharomyces cerevisiae var. *boulardii*	YGP/30	Culture collection of Copenhagen University
S. cerevisiae KVL 013	YGP/30	Culture collection of Copenhagen University

*BHI: Brain Heart Infusion; NA: Nutrient Agar; YGP: Yeast Glucose Peptone.
[a]Lund et al., 2000; [b]Stenfors and Granum, 2001; [c]Turnbull et al., 1979; [d]Agata et al., 1994; [e]Thorsen et al., 2010; [f]Lindegaard Pedersen et al., 2012.