Soya bean tempe extracts show antibacterial activity against *Bacillus cereus* cells and spores

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Running headline: *Bacillus cereus* inactivation by tempe

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ABSTRACT

Aims: Tempe, a *Rhizopus ssp.* fermented soya bean food product, was investigated for bacteriostatic and/or bactericidal effects against cells and spores of the food-borne pathogen *Bacillus cereus*.

Methods and results: Tempe extract showed a high antibacterial activity against *Bacillus cereus* ATCC 14579 based on optical density and viable count measurements. This growth inhibition was manifested by a 4 log CFU ml^{-1} reduction, within the first 15 min of exposure. Tempe extracts also rapidly inactivated *Bacillus cereus* spores upon germination. Viability and membrane permeability assessments using fluorescence probes showed rapid inactivation and permeabilization of the cytoplasmic membrane confirming the bactericidal mode of action. Cooked beans and *Rhizopus* grown on different media did not show antibacterial activity, indicating the unique association of the antibacterial activity with tempe. Subsequent characterization of the antibacterial activity revealed that heat treatment and protease addition nullified the bactericidal effect, indicating the proteinaceous nature of the bioactive compound.

Conclusions: During fermentation of soya beans with *Rhizopus,* compounds are released with extensive antibacterial activity against *B. cereus* cells and spores

Significance and Impact of Study: The results show the potential of producing natural antibacterial compounds that could be used as ingredients in food preservation and pathogen control.

KEYWORDS

Tempe, antibacterial, activity, *Bacillus cereus*, spores

INTRODUCTION

Tempe is the collective name for a sliceable mass of pre-cooked fungal fermented beans, cereals or some other food processing by-products bound together by the mycelium of a living mould (mostly *Rhizopus* spp.). Yellow-seeded soya beans are the most common and preferred raw material to make tempe. Traditional manufacture of tempe includes two major steps, namely a soaking process of the raw beans, where beans are acidified by natural occurring lactic acid bacteria. This is followed by a fungal fermentation initiated by an added mould starter culture. The purpose of the fermentation is not as much to enhance preservation, but rather the modification of ingredients and an increase of the nutritional properties due to enzymatic activity (Nout and Kiers 2005).

Previous investigations by Wang et al. (1969) showed that tempe contains antibacterial properties against some Gram-positive bacteria. These were associated with the mould *Rhizopus oligosporus.* In 1992, an antibiotic substance produced by *Rhizopus oligosporus* grown in culture broth was purified (Kobayasi et al. 1992) and found to be active against *Bacillus subtilis* vegetative cells. Neither further investigations of this antibacterial effect, nor the effect of tempe extracts on *Bacillus* spores have been published since.

The present paper deals with the antibacterial effect of soya bean tempe on *Bacillus cereus* vegetative cells and spores. *B. cereus* is a Gram-positive, spore forming bacterium, able to cause two types of food poisoning: the diarrhoeal type by enterotoxin production in the small intestine and the emetic type by cereulide, a toxin produced during growth in food (Kotiranta et al. 2000). *B. cereus* is ubiquitous in nature and frequently isolated from soil and plants. As a common inhabitant of soils, the organism can easily be transmitted to vegetation and hence to foods (Notermans and Batt 1998; Stenfors Arnesen et al. 2008).

The potential use of natural antimicrobials such as bacteriocins, for the improvement of microbial quality and safety of food has stimulated intensive research efforts in recent years

(Galvez et al. 2007; Settanni and Corsetti 2008). An antibacterial compound produced by tempe processing may be of considerable interest, due to its natural food-based origin. Inhibition of spoilage caused by outgrowth of bacterial spores may be an additional target for application, because of the resistance of spores to several preservation techniques. Pathogenic organisms are also developing more resistance to conventional antibiotics. This problematic trend has generated an increased interest in the pharmacological application of antimicrobial peptides to cure infections (Epand and Vogel 1999). Food-based antibacterial compounds may not only be important for food preservation but can possibly also be used to control pathogens and prevent human infections (Cotter et al. 2005).

In the present study the antibacterial activity of tempe is investigated with *Bacillus cereus* as the target organism. The activity is not only tested against vegetative cells, but also against spores. Furthermore, the origin of the active component by testing the intermediate stages of processing and fermentation strains separately is determined. Experiments are performed to give details about the mode of action and characteristics of the active component

MATERIALS AND METHODS

Tempe processing

Dry-dehulled full-fat yellow-seeded soya beans (*Glycine max*) were soaked overnight in tap water at 30ºC. Tap water was enriched with 10% naturally acidified soaking water from previous soya bean soaking steps, according to the "backslop" procedure (Nout et al. 1987). After overnight incubation soya beans were rinsed with tap water and cooked in fresh tap water for 20 min (ratio soaked beans: water 1:3) and cooled by evaporation of adhering moisture at room temperature. Soya beans were inoculated with a sporangiospore suspension of *Rhizopus microsporus* var. *microsporus* (LU 573). This suspension was prepared by scraping off the sporangia from pure cultures grown on malt extract agar slants (CM59; Oxoid, Basingstoke, UK) for 7 days at 30ºC, in PPS (0.85% NaCl and 0.1% peptone solution). After inoculation with the sporangiospore suspension (10 ml kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans), the soya beans (batches of 450 g) were packed in hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated at 30ºC for 24, 48, 72, 96 and 120 h.

Soya extract preparation

Fermented soya beans (tempe) and cooked soya beans, were freeze-dried and ground passing through a 0.5 mm sieve (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) and were stored at -20ºC until further processing.

Freeze-dried products were suspended in distilled water (60 g l^{-1}) and stirred with a magnetic stirrer for 3 h at room temperature. The pH was continually checked and adjusted to pH 8.0 with 1 M NaOH. In order to obtain clear supernatants, the soluble extract was obtained by three consecutive centrifugation steps (10 min, 10000 x *g*, 20ºC). Supernatants were freeze-

dried and soluble dry matter was stored at -20ºC and used as soya bean soluble dry matter in experiments.

Mould extract preparation

Rhizopus microsporus var. *microsporus* (LU 573) was grown on different liquid and solid media, to test the antibacterial activity of the mould biomass. *Rhizopus* sporangiospores were inoculated in malt extract broth (MEB, CM59; Oxoid, Basingstoke, UK) and mineral medium (MIB) containing NH_4Cl (5mM), D-glucose (25 mM), $MgSO_4.7H_2O$ (1.3 mM), $ZnSO_4.7H_2O$ (0.3 mM), $MnSO_4.4H_2O$ (0.2 mM), $FeCl.6H_2O$ (70 µM), $CuSO_4.5H_2O$ (40 µM) and EDTA (1.2 mM) in a 0.01 M K-phosphate buffer and grown for 4 days in a shaking incubator (30°C, 200 rpm). Mycelium was collected on a folded paper filter (no. 311651; Schleicher & Schuell GmbH, Germany) and washed three times with PPS by centrifugation (10 min, 3000 x *g*, 20°C). The pellet was freeze-dried and stored at -20°C until use. After removal of the mould biomass, the post-growth media were also freeze-dried and prepared for the activity assay, to test the effect of released fungal components. *R. microsporus* was also grown on plates of Malt Extract agar (MEA) and cooked soya bean agar (CSBA) (30 g l^{-1} freeze-dried cooked soya beans powder and 15 g l^{-1} agar) for 4 days at 30°C, followed by scraping off the mycelium from the agar plates and freeze drying of the mycelium. The collected mycelium extract and post-growth media extracts were prepared in the same way as described for the soya bean extracts.

Bacteria and culture conditions

B. cereus strain ATCC 14579 was used for investigation of the tempe antibacterial effect. Stock cultures were stored at -80ºC in 25% glycerol. Prior to experiments, strains were

inoculated in Brain Heart Infusion broth (BHI, 237500, Becton Dickinson, France) and grown for 16-18 h at 30ºC in a shaking incubator (200 rpm).

Spores were obtained from an overnight culture of *B. cereus* ATCC14579 grown in tubes with 5 ml Luria Broth (LB; 241420, Becton Dickinson, France) at 30°C and 200 rpm rotary shaking. Then cells were inoculated in maltose sporulation medium (MSM) based on the sporulation medium used by Schaeffer et al. (1965) fortified with sporulation elements of the defined medium for *B. cereus* and maltose to increase the yield. The MSM contained Nutrient Broth (8 g l^{-1}; NB, 234000, Becton Dickinson, France), maltose (10mM), $(NH_4)_2SO_4$ (5 mM), $MgCl_2$ (1 mM), $Ca(NO_3)_2$ (1 mM), $FeSO_4$ (1 mM), $MnSO_4$ (66 µM), $ZnCl_2$ (12.5 µM), $CuCl_2$ (2.5 µM), Na_2MoO_4 (2.5 µM) and $CoCl_2$ (2.5 µM). Sporulation was performed in 50 ml MSM in 250 ml Erlenmeyer flasks, at 30°C and 200 rpm rotary shaking. Sporulation efficiency was determined by microscopical observation and droplet plating before and after heating of sporulating cultures. In order to obtain spore batches containing (>95%) spores only, as controlled microscopically, spores were washed in 10 mM potassium phosphate buffer (pH 7.4), at least 10 times during the first 3 weeks, before starting any further experiments. Spore suspensions were stored at 4ºC and washed weekly to prevent spontaneous germination.

The antibacterial spectrum of tempe was tested on the following strains: *Bacillus cereus* ATCC 10987, NIZO B437, PAL 20, PAL28, 55, *Bacillus weihenstephanensis* DSM11821T and *Bacillus subtilis* B20010. All strains were inoculated in BHI broth and grown for 16-18 h at 30ºC with rotary shaking (200 rpm) before experiments.

Antibacterial activity assay

Two different methods were used: (a) monitoring bacterial biomass by optical density and (b) viable count enumeration. In both methods, soya bean dry matter (20 g l^{-1}) was dissolved in distilled water by mixing for 3 h on a head-over-tail rotator, followed by centrifugation (10

min, 16000 x g, 20°C) and filter sterilization (0.22 µm, FP30/0.2CA-S, Schleicher & Schuell GmbH, Germany). The pH of the extract was around 7. The sterile filtrate was diluted 1:1 in double concentrated BHI to achieve a 10 g l^{-1} extract in growth medium or was diluted further in BHI.

For optical density (OD) measurement 50 µl of a suspension containing 10^6 CFU ml^{-1} vegetative cells or spores were inoculated in 200 µl of BHI with or without tempe extract, in 96-well microtiter plates in triplicate. For each experiment, inoculation levels were verified by viable counts on BHI-agar. Microtiter plates were incubated in a spectrophotometer plate reader (SpectraMax Plus 384, Molecular Devices Ltd, United Kingdom) at 30ºC up to 24 h. Optical density was measured every minute at 600 nm. Prior to each measurement the plate was shaken for 45 s to ensure homogeneity and optimal aerobic growth conditions. The antibacterial activity is expressed as the growth delay in h:

$$Growth\ delay\ (h) = t_{OD=0.6,\ exp} - t_{OD=0.6,\ control}$$

Growth delay is defined as the time *B. cereus* growing in BHI with tempe extract (exp) needed to reach an OD of 0.6 minus the time *B. cereus* in BHI (control) needed to reach this OD-point. Data represent means and standard deviations of triplicate measurements.

During viable count enumeration experiments, BHI broth with or without tempe extract was inoculated in the same ratio medium/inoculation (4:1) as performed in the optical density measurement and was incubated at 30ºC with continuous shaking (200 rpm). Samples were taken at different time points, diluted with PPS and plated in duplicate on BHI-agar.

Microscopic observations

During viable count enumeration, *B. cereus* cells and spores were investigated microscopically. Bacterial cells were collected by centrifugation (3 min, 1520 x *g*, 20ºC), suspended in PPS and observed by phase-contrast microscope (magnification 1000x).

For viability determination, a fluorescence double staining technique was used. The green fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI) (Live/Dead *Bac*light Viability Kit, InVitrogen L7012) were mixed in a 1:1 dye mixture. *B. cereus* cells or spores were re-suspended to 10^8 CFU ml^{-1} in a BSA-saline solution (2.5 g l^{-1} bovine serum albumin with 0.15 M NaCl); 1 ml of cell suspension was mixed with 2 µl dye mixture and incubated in the dark on ice for 15 min. The stained *B. cereus* cells were observed with a fluorescence microscope.

Effect of pH, enzymes and heat on antibacterial activity

Tempe extracts were prepared as described for the antibacterial activity assays. Different treatments were performed as follows.

To investigate any inhibitory effect of pH, the extracts were adjusted by addition of 1M NaOH or 1M HCl from pH 2.0 up to pH 9.0. For low pH's (2.0, 3.0 and 4.0) the tempe extract solution was adjusted with 1M HCl, mixed for an hour, centrifuged and supernatant was neutralized again to pH 7 with NaOH before antibacterial activity was measured.

Tempe extracts were incubated with different enzymes of ample concentration (10 mg ml^{-1}) at 30ºC with continuous shaking (200 rpm) for an extended period of 4 h to allow degradation of all susceptible material. The following enzymes were used: Pronase E (P5147), Proteinase K (P8044), Trypsin (T0134), α-Chymotrypsin (C4129) and Protease type XII (P2143) all from Sigma-Aldrich. Enzymes were not inactivated, because *B. cereus* growth was not affected by their presence as confirmed by control experiments with enzyme solutions only (data not shown).

Tempe extracts were heated for 20 min at 20, 30, 40, 50, 60, 70, 80, 90 and 100°C in a water bath. After heating the samples were immediately cooled down till 4°C, followed by measurement of their antibacterial activity.

After the treatments mentioned above, tempe extracts were centrifuged and diluted with BHI as done in antibacterial activity assay. Then they were inoculated with *B. cereus* and the antibacterial activity was measured by optical density growth measurements.

RESULTS

The impact soya bean extracts on *B. cereus* vegetative cells growth.

Growth of *B. cereus* was assessed in BHI with or without addition of soya bean extracts. Figure 1A shows the optical density increase during 24 h incubation at 30ºC and Figure 1B shows the viable count enumeration of *B. cereus* using the same soya beans extracts performed simultaneously. The growth of *B. cereus* in BHI with or without addition of 10 g l^{-1} cooked beans was similar, whereas the growth of *B. cereus* in BHI with 10 g l^{-1} tempe added showed a growth delay of 12.31 h ± 0.22 (mean ± standard deviation) (Figure 1A) and a reduction in viable count of 3.7 log CFU ml^{-1} (Figure 1B). This reduction in viable count occurred within the first 30 min of measurement, indicating a remarkable bactericidal effect. *B. cereus* viability was also analyzed microscopically using fluorescent live/dead staining. After 15 min of incubation *B. cereus* cells in BHI with added tempe extract were fluorescent red-stained which means that the PI stain entered the cells indicative of membrane damage (data not shown).

After 24 h incubation of *B. cereus* in BHI with added tempe extract, lower numbers of viable cells were reached (Figure 1B). Microscopic observations however showed that *B. cereus* formed long chains of rods in all three BHI with added tempe extracts samples compared to cooked and BHI without addition of tempe. This could result in less colonies on the agar plates and an underestimation of the viable cell count after 24 h. This aggregation of bacterial cells was not observed after 15 minutes.

The impact of soya bean extracts of the tempe processing stages on *B. cereus* vegetative cells growth.

During tempe processing, antibacterial activity of intermediate soya products was observed using the optical density assay. Growth delay caused by soya and tempe extracts of the

successive stages of the processing are shown in Figure 2A. Growth delays caused by raw, soaked and cooked soya beans, *i.e.*, soya extracts of the processing steps before mould inoculation, were negligible. No effect of the soaking step, during which lactic acid bacteria actively develop, was observed. After inoculation with *R. microsporus* and incubation for 24 h the growth delay of *B. cereus* had increased up to 9.36 ± 0.5 h (mean ± standard deviation). Longer fermentation times resulted in less growth delay decreasing to 5.33 ± 0.08 h (mean ± standard deviation) after 120 h of fermentation.

The antibacterial activity of *R. microsporus* grown on different media and their post-growth media was also tested (Figure 2B). *R. microsporus* grown on the different media, MEB, MIB, MEA and CSBA showed a growth delay less than 1.8 h, whereas the tested tempe extract showed a delay of 14.9 ± 2.36 h. Also the post-growth media showed no activity towards *B. cereus*.

Effect of soya bean extracts on germination and outgrowth of *B. cereus* spores

B. cereus spores (log 9 CFU ml^{-1}) were incubated with and without soya bean extracts and monitored for their germination and growth. Figure 3A presents the results of the OD measurement. All samples showed a similar germination, as was manifested by an OD drop within 1 h reflecting transition from the phase bright dormant phase into the phase dark germinated phase (60% loss of optical density represents a germination of 100%). After germination the spores in BHI with or without added cooked beans extracts showed a normal pattern of outgrowth of vegetative cells. Spores germinated in BHI with added tempe extract showed a long “lag” phase after germination, followed by an increase of optical density after 8 h indicating resumption of growth. The two tempe concentrations showed similar growth inhibition of *B. cereus*. Figure 3B presents development of viable counts of *B. cereus* after inoculation of spores in BHI with or without added tempe extract. A reduction of viable

counts of 3.7 log CFU ml^{-1} within 2 h was observed, with the fastest decrease of viable cells within the first 30 min. This experiment also revealed similar effects of the different concentrations of tempe extracts used.

Spore germination and outgrowth in BHI with or without added tempe extract was monitored using phase contrast and fluorescence microscopy (Figure 4). After 15 min of incubation with and without tempe extract, dormant spores changed from phase bright to the phase dark stage. As was also shown in the OD-measurement (Figure 3A), tempe did not hamper the first stage in spore germination. Phase contrast and fluorescence microscopy showed that the phase dark spores with tempe were stained red, whereas the spores germinated in normal BHI were stained green. The red color indicates the red fluorescent membrane-impermeant dye PI to have entered the germinated spores signifying membrane damage by the incubation with tempe extract. After 2 h of incubation in BHI with added tempe extract, all spores still remained phase dark and appeared red-fluorescent (PI-stained) after live-dead staining, whereas the spores that germinated in BHI had developed rod-shaped cells that appeared green-fluorescent (SYTO 9-stained) after staining.

Tempe antibacterial spectrum

Antibacterial activity of tempe was tested against different *Bacillus spp.* at two extraction pH's: pH 7 and pH 8. Table 1 shows tempe to exert an antibacterial effect on all tested *B. cereus* strains and also on *B. weihenstephanensis* and *B. subtilis*. Tempe extracts at pH 8 showed a longer growth delay than extracts obtained at pH 7; extracts of tempe fermented for 24 h showed a longer growth delay than the tempe fermented for 48 h for all tested strains. No growth of any tested *Bacillus* strain occurred within the 22 h of the measurement in extracts of tempe fermented for 24 h and adjusted to pH 8.

Characterization of the inhibitory agent

Tempe extracts were tested for their antibacterial activity after pre-incubation at different pH values, exposure to a range of enzymes and after heat treatment (Table 2). After pH adjustment of the tempe extracts, a growth delay of *B. cereus* could only be observed at pH 6 and higher, with highest inhibition activity at at pH 8 and 9.

After treating tempe extracts with Pronase E or Proteinase K, all antibacterial activity was lost. The activity was partly susceptible to the Protease type XIII and resistant to Trypsin and α-Chymotrypsin.

After heat treatment exceeding 60°C, the antibacterial activity was gradually lost. Heating up to 60°C resulted in an activity loss of about 20% and 60% in extracts of tempe fermented for 24 h and 48 h, respectively. Cooking led to complete loss of activity for the extract of 48 h fermented tempe, whereas the 24 h sample retained 30% of its activity. Notably, the 24 h fermented tempe was more active than the 48 h fermented tempe, and was also more resistant to low pH and higher temperatures.

DISCUSSION

This study describes the antibacterial effect of soya bean extracts of intermediate stages of processing and fermentation of tempe on *Bacillus cereus* vegetative cells and spores. The antibacterial activity was found to be specific for tempe extracts and was sensitive to heat, low pH and proteases, indicating its proteinaceous nature.

Two antibacterial activity assays were performed, using optical density monitoring and viable count enumeration (Figure 1). Both showed a rapid bactericidal effect by a long growth delay of 12 h and a reduction in viable count of 3.7 log CFU ml^{-1} of the *B. cereus* cells after addition of tempe extract. After several hours a re-growth of the bacteria was observed in both experiments and could be explained by either degradation of the active component, or a surviving subpopulation. Conceivably, enzymes produced by *B. cereus* or produced by the mould during fermentation could be responsible for this reduction of antibacterial activity of the tempe extracts. Indeed, when *B. cereus* overnight culture supernatants were added to tempe extracts, the antibacterial activity of tempe was reduced to 10% of its initial value (data not shown). It is known that *Bacillus spp*. can produce a diversity of soluble extracellular enzymes (Priest 1977), including proteases that may inactivate the active component(s).

Experiments with *B. cereus* spores showed that tempe could inactivate spores upon their germination. The optical density data (Figure 3A) demonstrate the transition of phase bright spores into phase dark spores, followed by outgrowth of the vegetative cells. The spores in BHI with added tempe extract showed a growth delay of around 6 h compared with the spores growing in control BHI, which suggests that only a small surviving subpopulation of spores was able to grow. Fluorescence microscopy experiments showed that after addition of tempe extract (Fig. 4B) the phase dark spores (after germination) were stained red by propidium iodide, which indicates that tempe extracts induced considerable membrane damage to the germinated spores as was observed also with vegetative cells. The effect of tempe on dormant

spores could not be measured as the tempe extracts already triggered the germination of the spores to the phase dark stage. Mechanistic information about effects of antibacterial compounds against spores is limited. A recent study, concerning the well known antibacterial compound nisin, showed nisin to act against germinated spores of *Bacillus anthracis* and not against dormant spores. The mode of action of nisin responsible for outgrowth inhibition of *B. anthracis* spores appeared to involve inhibition of the oxidative metabolism and dissipation of the membrane potential, indicative of disruption of membrane integrity (Gut et al. 2008). Although our results also point to membrane targeting of the antibacterial activity of tempe extracts, the active component is different from nisin, based on its sensitivity to heat and low pH, and its inactivation by a range of proteases.

In the research of Wang et al.(1969) and Kobayasi et al. (1992) antibacterial activity of *Rhizopus oligosporus* grown in culture broth was investigated. In contrast, we were not able to determine antibacterial activity of *Rhizopus microsporus* grown in culture broth, even when it had been grown on cooked soya bean agar. However, after the fermentation of *R. microsporus* on soya beans a high bactericidal activity was observed. It is conceivable that the antibacterial activity is produced by microorganisms present in tempe, i.e. *Rhizopus microsporus* and LAB bacteria. This indicates on the one hand, that the antibacterial activity is only produced *in situ*, but on the other hand, it may indicate that degradation products of soya proteins are responsible for the antibacterial effect of tempe, thus pointing to an indirect role of the indicated microorganisms. Notably, for milk derived peptides such as bovine lactoferricin from lactoferrin and several peptides from casein, a strong antimicrobial activity was observed after microbial hydrolysis (López-Expósito and Recio 2008). Further characterization of the antimicrobial activity will shed light on this hypothesis.

To conclude, tempe extracts display antibacterial activity against *B. cereus* cells and spores by targeting the membrane of the bacteria. This bactericidal activity is released during

fermentation of soya beans only, whereas extracts of raw, soaked and cooked soya beans, and *Rhizopus microsporus* grown in culture media did not give any antibacterial activity. The tempe antibacterial activity was found to be heat, low pH and protease sensitive, suggesting its proteinaceous nature. Further research will be required to identify the chemical nature of the antibacterial component(s) and its bio-functionality in terms of food preservation and pathogen control.

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Table 1:

Growth delay (h) based on optical density of *Bacillus* strains exposed to tempe extracts.

	Growth delay (h)			
	Tempe, 24 h*		Tempe, 48 h*	
Strain	pH 7†	pH 8†	pH 7†	pH 8†
B. cereus (ATCC 14579)	9.2 ± 0.4	> 16.6	6.3 ± 0.1	> 16.6
B. cereus (B437)	11.7 ± 4.4	> 15.1	5.3 ± 0.4	13.0 ± 2.9
B. cereus (PAL 20)	> 16.7	> 16.6	10.8 ± 1.1	13.9 ± 3.9
B. cereus (PAL 28)	> 16.8	> 16.7	6.6 ± 0.3	10.6 ± 0.0
B. cereus (55)	13.7 ± 0.4	> 16.4	7.1 ± 0.3	> 16.4
B. weihenstephanensis (DSM 11821T)	12.6 ± 3.1	> 15.6	7.5 ± 0.0	13.9 ± 2.5
B. cereus (ATCC 10987)	> 16.6	> 16.3	15.2 ± 2.0	> 16.3
B. subtilis	10.9 ± 2.2	> 12.5	> 12.5	> 12.5

Growth delay is expressed as mean ± standard deviation;

*Tempe extracts 10 g/l in BHI of 24 and 48h fermented tempe

† Growth delay measured at pH 7 and pH 8

> means that the growth delay is longer than the total measurement period (22 h).

Table 2:

Effect of pH, enzyme and heat treatments on growth inhibition activity of tempe extracts against *B. cereus* ATCC 14579

Treatment	Activity (%)	
pH	Tempe, 24h*	Tempe, 48h*
2	0.4 ± 0.8	ND
3	2.0 ± 0.8	ND
4	0.5 ± 0.8	ND
5	17.3 ± 1.7	15.8 ± 4.5
6	54.2 ± 2.3	16.9 ± 1.3
7.1 (control)	100 ± 7.8	100 ± 4.7
8	> 100†	> 100†
9	> 100†	> 100†
Enzyme		
No enzyme (control)	ND	100 ± 14.1
Pronase E	ND	-1.8 ± 0.8
Proteinase K	ND	-3.5 ± 0.3
Protease XIII	ND	67.6 ± 5.4
Chymotrypsin	ND	101.0 ± 9.2
Trypsin	ND	> 100[2]
Heat (°C, 20 min)		
4	100.0 ± 5.3	100 ± 12.1
20	97.6 ± 5.0	99.5 ± 12.6
30	106.7 ± 4.9	101.7 ± 10.4
40	118.2 ± 6.0	99.9 ± 12.8
50	131.9 ± 6.2	73.9 ± 10.4
60	83.9 ± 5.5	42.3 ± 9.1
70	47.9 ± 4.9	28.8 ± 8.3
80	41.1 ± 5.5	18.1 ± 8.5
90	33.6 ± 6.9	1.8 ± 7.2
100	32.2 ± 6.5	-4.0 ± 7.1

* Tempe extracts 10 g/l of 24h and 48h fermented tempe

† No growth observed within measurement period.

Activity is expressed in % activity after treatment compared with the tempe without any treatment in the specific experiment. Activity is expressed as mean ± standard deviation; ND: not determined

FIGURE CAPTIONS

Figure 1: Influence of tempe extracts on vegetative cells of *B. cereus* 14579.

A: Growth of *B. cereus* 14579 by optical density in BHI (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square) and tempe 10 g l^{-1} fermented for 24 h (closed triangle). For clarity of the figure, data points at 15 min intervals are shown.

B: Viable count enumeration of *B. cereus* 14579 growth in BHI (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square) and tempe 10 g l^{-1} fermented for 24 h (closed triangle). The dotted lines are predictions of the growth between 9 and 26 h.

Figure 2: Growth delay measured by optical density of *B cereus* 14579 after exposure to several extracts.

A: Growth delay of *B. cereus* 14579 after exposure to extracts (10 g l^{-1}) of several soya bean products during tempe processing. X-axis represents the several stages during tempe processing, respectively: raw soya beans (raw), soaked soya beans (soak), cooked soya beans (cook), tempe fermented for 24 h (tempe, 24h), tempe fermented for 48 h (tempe, 48h), tempe fermented for 72 h (tempe, 72h), tempe fermented for 96 h (tempe, 96h), tempe fermented for 120 h (tempe, 120h).

B: Growth delay measured of *B. cereus* after exposure to mould biomass grown in different media, and to corresponding post-growth media. The concentration of the tempe (fermented for 24 h) extract is 10 g l^{-1} and of the mould extract 1 g l^{-1}. MEB: malt extract broth; MIB: Mineral medium; MEA: Malt extract agar; CSBA: Cooked soya bean agar; PG: Post growth medium; LU573: inoculated with *Rhizopus microsporus* LU 573.

Figure 3: Influence of tempe extracts on spores of *B. cereus* 14579.

A: Germination and outgrowth of spores by optical density in BHI (closed square), cooked soya beans 10 g l^{-1} (open triangle), tempe 1 g l^{-1} fermented for 48 h (closed circle), tempe 10 g l^{-1} fermented for 48 h (open square), tempe 1 g l^{-1} fermented for 24 h (open circle) and tempe 10g l^{-1} fermented for 24 h (closed triangle). For clarity of the figure, data points at 15 min intervals are shown.

B: Viable count enumeration of *B. cereus* spores behaviour during 3 h incubation at 30ºC. Control growth in BHI (closed square), tempe 1 g l^{-1} fermented for 48 h (closed circle) and tempe 10 g l^{-1} fermented for 48 h (open square)

Figure 4: Spore germination and outgrowth in BHI, as affected by added tempe extracts. Phase contrast (left) and fluorescence (right) microscopy photographs of *B. cereus* ATCC 14579 spores in BHI (A) and BHI supplemented with 1 g l^{-1} tempe extract (B) after 0 min, 15 min and 2 h. For fluorescence microscopy the green fluorescent SYTO 9 and the red-fluorescent propidium iodide (PI) were used.

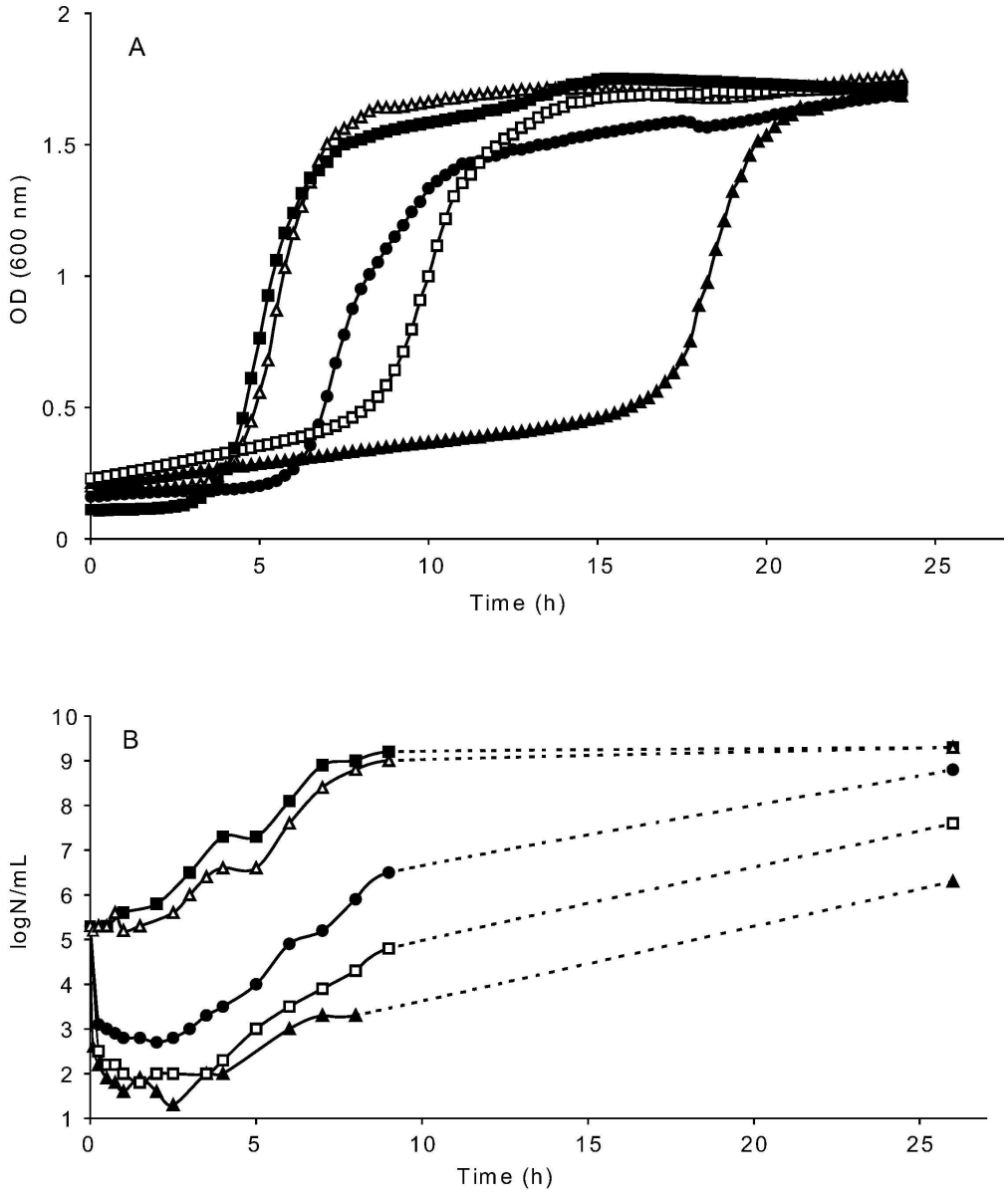

Figure 1
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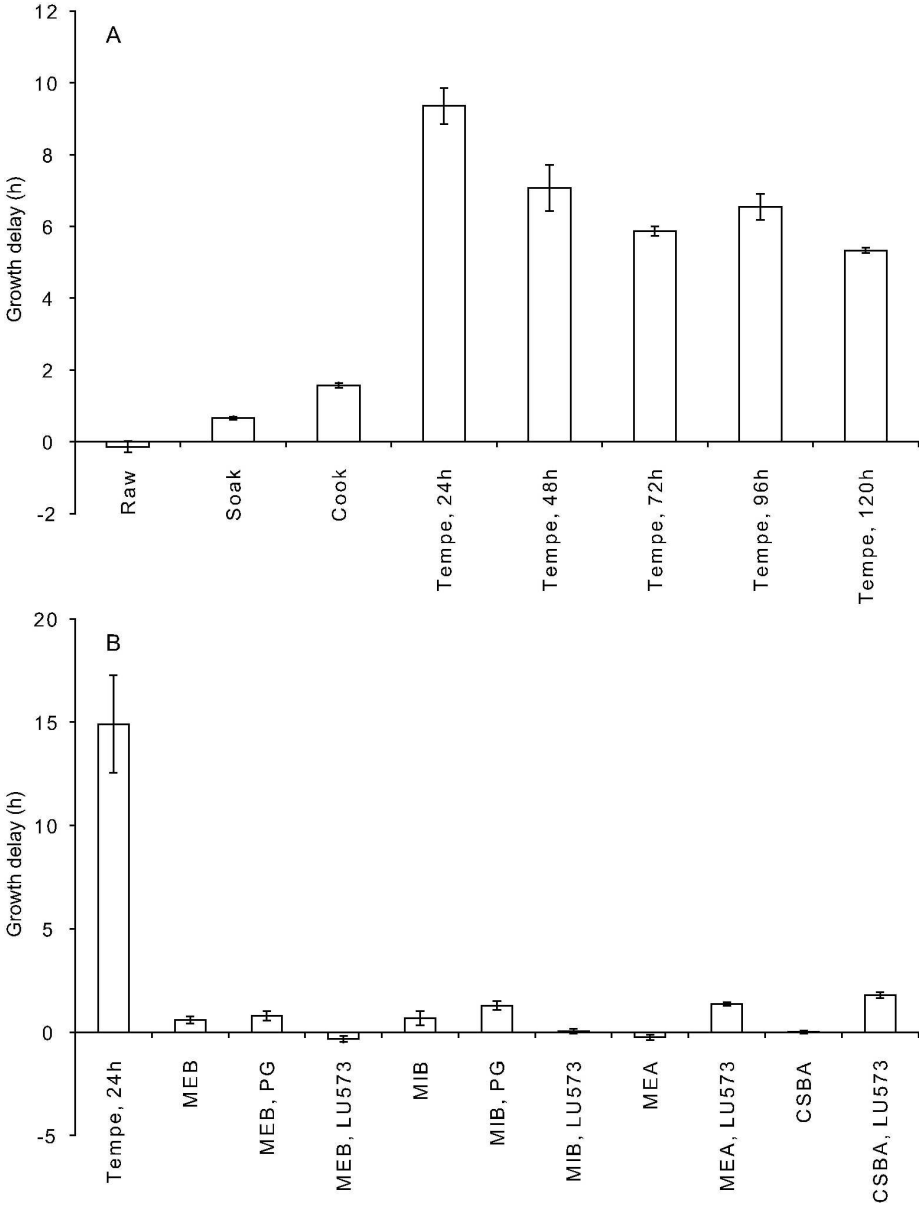

Figure 2
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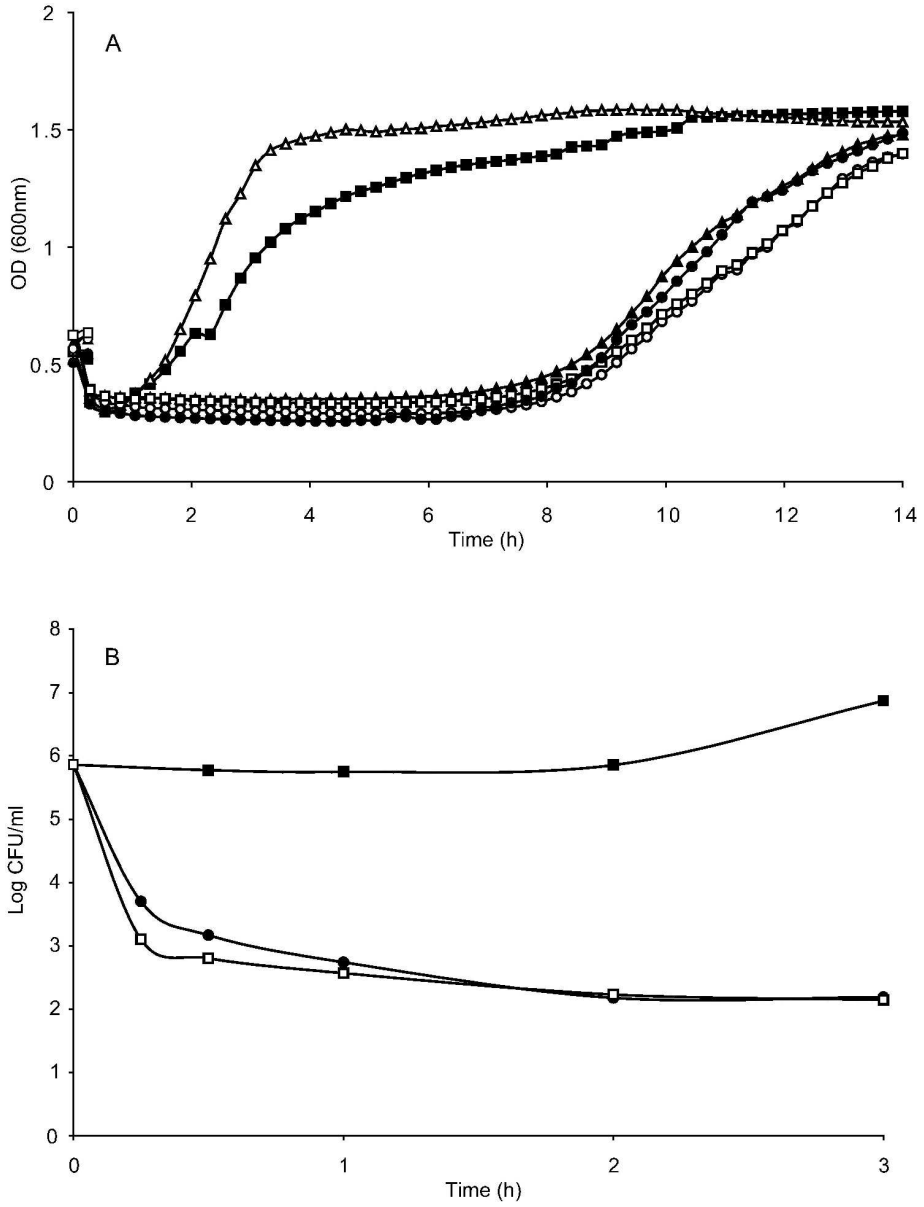

Figure 3
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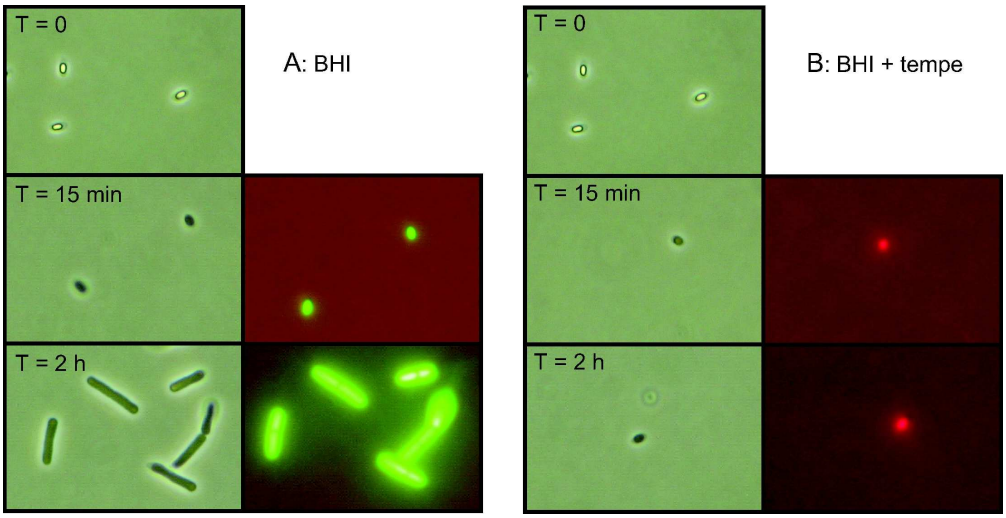

Figure 4
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