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Research Article

Designing a SYBR Green Absolute Real time PCR Assay for Specific Detection and Quantification of *Bacillus subtilis* in Dough Used for Bread Making

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Abstract

In this present study, a SYBR green based real time PCR assay has been developed for specific detection and quantification of *Bacillus subtilis* in dough used for bread making. New primer pairs were designed to amplify a 212 base pair fragment of the *aprE* gene. Specificity of these primer pairs was confirmed with conventional and real time PCR methods. Standard curves constructed using the threshold cycle (C_T) versus copy numbers of *B. subtilis* showed good linearity for reference standards of cloned insert (R^2=0.999, slope=-3.035) and also induced contaminated dough (R^2=0.988, slope=-3.142), and the melting temperature (T_m=82.2 ^oC) was consistently specific for the amplicon. Limits of detection were 200 and 2000 colony forming units (CFUs) per ml or g of these samples, respectively. This real time PCR offers a fast tool with high sensitivity and specificity for detection and quantification of this rope-forming pathogen in dough used for bread making.

***Keywords*:** Real-time PCR, *Bacillussubtilis*, new primer pairs, contaminated dough

Introduction

Bacillus subtilis is a gram positive member of the genus*Bacillus*, which is rod shaped, catalase positive and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions (Jay et al., 2005; McMeekin, 2000). In recent years, many researchers have focused on this bacterium due to its association with foodborne illnesses and also as a consequence of its thermal tolerance, especially concerning its reported resistance to commercial baking conditions and spore forming ability in food products (Nicholson et al., 2000; PAVIC et al., 2005). The importance of *B. subtilis* in rope spoilage of bread has also been confirmed. Rope spoilage is the most important bacterial spoilage of bread that is usually caused by *Bacillus* spp., especially *B. subtilis*(Rosenkvist and Hansen, 1995; Sorokulova et al., 2003). Due to the large number of species and the often incomplete descriptions of a number of newly reported species within the genus *Bacillus*, it is very difficult to determine the species types and therefore some recent works have focused on optimizing more efficient culture independent methods and detection strategies to overcome this problem (Fernández-No et al., 2011; Liu, 2010; Sadeghi et al., 2012).

Rapid detection and quantification of microbial species are crucial to improve food safety by developing effective preventive and/or adjustment measures. During the past 30 years, tremendous insights have been gained on how microorganisms spread and cause various diseases (Justé et al., 2008; Martínez et al., 2011). As well as conventional PCR, the advent of SYBR green technology has offered the ability to simultaneously detect and quantify DNA from specific targets using rapid real time PCR (Levin, 2010). The use of shortened target DNA sequences in real time PCR results in more efficient amplification than standard PCR where amplicons are required to be at least

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200 bp in length to allow detection by electrophoretic separation, and also allows reduced extension times (Fricker et al., 2007; Martínez et al., 2011).

In real time PCR, unlike in conventional PCR, the amplification is monitored continuously during the reaction, which permits the user to quantify the target faster. Real time PCR is performed in a closed tube system and requires no post-PCR manipulation of the samples, preventing PCR mix contamination. Real time PCR depends on the emission of an ultraviolet induced fluorescent signal that is proportional to the quantity of DNA that has been synthesized. The simplest, least expensive and most direct fluorescent system for real time PCR involves the incorporation of the SYBR green dye whose fluorescence under UV greatly increases when bound to the minor groove of double helical DNA. The quantification of the target amount in unknown samples can be estimated by generating a standard curve. The establishment of a standard curve using the quantitative real time PCR process is a key step in determining the copy number of a given target sequence. A perfect amplification reaction produces a standard curve with an efficiency of two, because the amount of target DNA should double during each cycle (Klein, 2002; Mackay, 2004; Postollec et al., 2011).

In the past decade, more researchers relied upon real time PCR studies using simple and less expensive SYBR green dye. A number of SYBR green real time PCR assays for detection and quantification of foodborne bacterial pathogens such as *Escherichia* (Jothikumar and Griffiths, 2002), *Mycobacterium*(O'Mahony and Hill, 2002), *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Vibrio*, *Aeromonas*, *Staphylococcus*, *Clostridium* and *Bacillus*(Fukushima et al., 2003), *Salmonella* and *Listeria* (Jothikumar et al., 2003), *Campylobacter*(Inglis and Kalischuk, 2004), *Vibrio*(Panicker et al., 2004), *Salmonella*(Nam et al., 2005), *Enterobacter*(Liu et al., 2006), *Clostridium*(Fenicia et al., 2007), *Plesiomonas*(Gu and Levin, 2008), *Klebsiella*(Sun et al., 2010) and *Staphylococcus*(Fusco et al., 2011) have been reported.

The objective of this study was to develop and evaluate a SYBR green absolute real time PCR method for the specific detection and enumeration of *B. subtilis* in culture medium and dough samples used for bread making.

Materials and Methods

Bacterial species, induced contamination and DNA extraction

Bacterial species (*Bacillus subtilis* ATCC 6633, *Bacillus amyloliquefaciens* ATCC 23350, *Bacillus cereus* ATCC 11778, *Bacillus licheniformis*ATCC 9789, *Bacillus* spp., ATCC 21832, *Staphylococcus aureus* ATCC 6538, *Escherichiacoli* ATCC 25922 and*Lactobacillus* spp., ATCC 8001) used in this study were purchased from American Type Culture Collection as vacuum dried cultures. These species, after activation, were plated into nutrient agar (Merck, Germany), incubated for 24-48 hours at 30-37 ^{o}C and then pure bacterial colonies were obtained (Jay et al., 2005). Wheat flour was prepared from a local flour mills factory. Chemicals (Merck, Germany), conventional PCR reagents (Fermentas, Lithuania), designed primers (MWG, Germany), DNA Extraction DNeasy kit (Qiagen, Germany), GeneJET Plasmid Miniprep Extraction kit (Thermo Scientific, USA), S.N.A.P. Gel Purification kit (Invitrogen, USA), Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, USA) and InsTAclone PCR Cloning kit (Thermo Scientific, USA) were also purchased and used.

Dough samples were prepared according to our previous study (Sadeghi et al., 2012). Then equal numbers of the mentioned bacterial species were used for contamination of the dough. 25g of dough was inoculated with bacteria at numbers of ca. 10^5 CFU/g of each species, separately (Blackburn, 2006; McMeekin, 2000).Qiagen genomic DNA extraction kit was used according to the manufacturer protocol to obtain genomic DNA from cultured cells. The extracted DNA from activated *B. subtilis* in nutrient broth culture was used as positive control in following PCR assays. Extraction of the total DNA from dough samples was performed as described by Merothe*t al.*(Meroth et al., 2003). The DNA extracted from artificially contaminated dough samples with related and unrelated species that may be naturally present in dough including *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis,Bacillus* spp., *S.aureus*, *E.coli* and *Lactobacillus* spp., were also used as negative controls in PCR tests.

Primer design, specificity evaluation and conventional PCR setting

Forward primer (5'-ACCATTGCGGTAGGTGCG-3') and reverse primer (5'-GCGTTTGTCCAAGTCGGG-3') were designed according to the *aprE* gene sequence published in the GeneBank (AJ539133). The target

gene encodes subtilisin toxin precursor and is a highly conservative and specific gene of *B. subtilis*(Sadeghi et al., 2012). The design of primers was performed using Primer Premier 5 (Premier Biosoft, USA) according to the recommendations of software. Computer simulation of different combinations of all the primer pairs was performed in order to find the best combination for development of the real time PCR test and the primers were then tested for specificity using BLAST (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Primer specificities were also assayed and assessed by cross reaction among mentioned related and unrelated bacterial species. Specificity of primer was further affirmed by carrying out melting peak analysis of real time PCR assays on the amplicon.

The conventional PCR tests were performed on a Corbett N15128 thermocycler (Australia). First the PCR was optimized for detection of *B. subtilis*. After evaluating the effects of different annealing temperatures (in the range of 50 to 60^{o}C), optimized PCR reaction (initial denaturation at 94^{o}C for 4 min, amplification for 35 cycles at 94^{o}C for 30 s, 54^{o}C for 45 s, 72^{o}C for 45 s and final extension of 7 min at 72^{o}C) was carried out in 20 µl final volume including 2 µl *Taq* polymerase with 2.5 units activity, 2.5 µl buffer 10x, 2.5 µl $MgCl_2$ at concentration of 25 mM, 0.5 µl of dNTPs mixture (10 Mm), 2 µl of each primer at concentration of 0.5 mM and 2 µl DNA with concentration of 100 ng per µl. Then PCR products were electrophoresed on 1.5% (w/v) agarose gel in TBE buffer with pH 8, stained by SYBR Safe DNA gel stain (Invitrogen, USA) and observed under UV light. Based on the *B. subtilis* target gene sequence, the amplified PCR products should be 212 bp. Finally, the PCR product was sent for sequencing to MWG Co. (Germany) and sequencing result was evaluated by BLASTN procedure with the available data in NCBI.

Constructing reference standard and generation of standard curve

The reference standards were constructed to detect *B. subtilis* with real time PCR. The 212 bp fragment of the *aprE* gene obtained using the designed primers was cloned. First this was achieved by amplifying the selected sequence of target gene using the conventional PCR and the presence of the specific amplified product was confirmed by electrophoresis. Purification of the amplified product was performed using the S.N.A.P. Gel Purification kit (Invitrogen, USA), according to manufacturer's recommendations. DNA manipulations were carried out according to standard protocols and instructions of InsTAclone PCR Cloning kit (Thermo Scientific, USA). These included ligation of the amplified 212 bp fragment of *B. subtilisaprE* gene with the TA cloning plasmid vector (pTZ57R/T), transformation of the construct into competent *E. coli* DH5α, screening of the positive clones and then plasmid DNA extraction by GeneJET Plasmid Miniprep kit (Thermo Scientific, USA) as illustrated in Fig. 1. The identity of the cloned insert was confirmed by direct sequencing, which was performed using the universal sequencing primer M13F (Dhanasekaran et al., 2010; Sun et al., 2010).

The standard curve was then generated using standard values obtained by 10 fold serial dilutions of the extract of the resulting plasmid harbouring the target insert. For this purpose, the amount of plasmid DNA was determined by a nanodrop spectrophotometer (Thermo Scientific, USA) at 260 nm. Then, 10 fold serial dilutions of the extract were prepared, ranging from 2×10^{8} to 2×10^{1} copies/ml based on the instruction available online at http://www.uri.edu/research/gsc/resources/cndna.html. Real time PCR of the standard dilution series was performed in triplicate as three separate tests. The standard curve was generated by plotting the threshold cycle (C_T) against the DNA amount (plasmid copies/ml) produced for the target sequence (Nicholson et al., 2000). Sensitivity was shown by applying these DNA dilution series of the reference strain to the real time PCR. For comparison of PCR amplification efficiencies and detection sensitivities among different experiments, slopes of standard curves were calculated by performing a linear regression analysis using the programme SPSS 12.0 for Windows (SPSS Inc., USA).

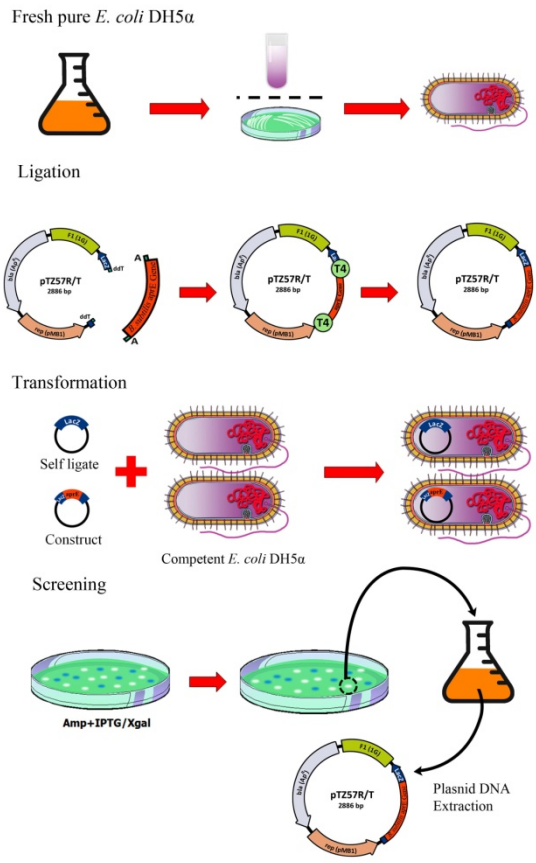

Figure 1. Schematic procedure of TA cloning used in this study for constructing the reference standard.

SYBR green real-time PCR amplification and melting curve analysis

Real time PCR was performed on the experimental samples and reference standards. The PCR was carried out using the Bio-RadCFX96 Touch™ machine (USA). Initially, primer pairs were run in PCR under the following conditions: Each 25 µl reaction mixture consisted of 12.5 µl Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA), 1 µl of forward and reverse primers (0.3 µM) and 2 µl of template DNA or extracted plasmid DNA from recombinant vector (100 ng). The standard protocol included one cycle at 95 ^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 30 s, 54^{o}C for 30 s and 72^{o}C for 45 s. Fluorescence was measured at the end of each extension step. In order to allow subsequent melting temperature (T_m) analysis, PCR products were heated to 95^{o}C for 1 s, cooled to 65^{o}C for 5 s and then reheated to 95^{o}C at a rate of 0.01 ^{o}C/s. The correct size of PCR products was verified by 1.5% gel electrophoresis and visualized with UV transillumination after SYBR Safe staining. The specificity of the developed assay was assessed with mentioned *Bacillus* and non *Bacillus* bacterial species that may be naturally present in dough. The identity of the PCR product from a sample was also confirmed by performing a melting curve analysis and comparing its T_m with positive control (Kennedy and Oswald, 2011; Martínez et al., 2011; Nam et al., 2005; Postollec et al., 2011). The amount of fluorescence emission in real time PCR correlates to the initial amount of target template during exponential phase. The T_m peaks of the products were calculated based on initial fluorescence curves by plotting negative derivative of fluorescence over temperature (Levin, 2010; Nam et al., 2005; Postollec et al., 2011).

Results

Specificity evaluation of designed primers in conventional PCR

Fig. 2 shows the PCR results for specific detection of *B. subtilis*by conventional PCR. Extracted DNA from all bacterial species as described in materials and methods, were examined as templates. Specificity of primers and absence of nonspecific products or primer dimers were tested by analyzing PCR products on a 1.5% agarose gel stained with SYBR Safe. Only the expected 212 bpamplicon could be observed after electrophoresis and no other bands were visible. To confirm the identity of the amplicon and further characterize the detailed sequence of the gene, the PCR product was sequenced and the result was verified by with BLASTN search to find any sequence similarities. There were no known non-target strain DNA sequences in the BLASTN database with homology to the sequence. Many researchers used this procedure to confirm the specificity of designed primers before their use in SYBR green real time PCR such as Nam *et al.*(Nam et al., 2005), Fenicia*et al.*(Fenicia et al., 2007), Sun *et al.*(Sun et al., 2010) and Martínez-Blanch *et al.*(Martínez-Blanch et al., 2009). The proposed method was able to identify correctly the target pathogen both in bacterial cultures and dough samples. Furthermore, the use of related and unrelated species as negative controls for detection of any amplifiable bacterial DNA and sequencing results, confirmed the specificity of the developed assay.

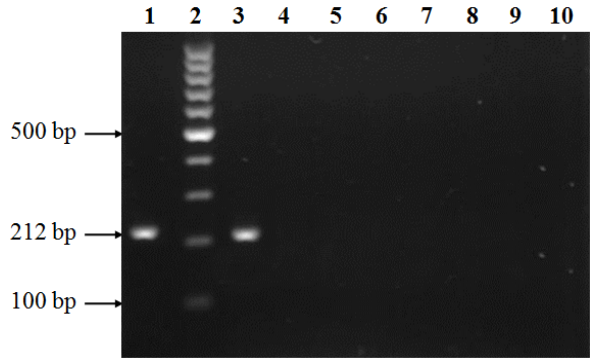

Figure 2. Agarose gel electrophoresis of PCR products obtained under optimized conditions for detection of *B. subtilis*(212 bp). Extracted DNA from cultured cells of *B. subtilis*in nutrient broth as positive control (lane 1), 100 bp DNA marker (lane 2), extracted DNA from inoculated dough with *B. subtilis*(lane 3), extracted DNA from dough artificially contaminated with B. amyloliquefaciens, B. cereus, B. licheniformis, Bacillus spp., S. aureus, E. coli and Lactobacillus spp., representing the negative controls with ca. 105 CFU/g of each bacterium (lanes 4 to 10, respectively).

Detection of *B. subtilis*by SYBR green based real time PCR

Nine samples tested with conventional PCR, including extracted DNA from cultured cells of *B. subtilis*in nutrient broth, extracted DNA from inoculated dough with *B. subtilis* and extracted DNA from dough artificially contaminated with *B. amyloliquefaciens*, *B. cereus, B. licheniformis, Bacillus* spp., *S.aureus*, *E.coli* and *Lactobacillus* spp., were analyzed for the presence of *B. subtilis* by real time PCR using SYBR green dye. DNA extracted from *B. subtilis* (cultured or artificially contaminated dough) yielded positive PCR products from the gene and all other samples were negative by both conventional and real time PCR. No false positive or false negative results were detected (Fig. 2 and Fig. 3). Specificity of the reaction was also confirmed by melting temperature analysis, which was constant for the amplicon obtained. *B. subtilis* evaluated in the present study, showed a constant T_m of 82.2 ± 0.2 ^oC equal to means ± standard deviation of ten separate tests (Fig. 3 b).

The unique T_m of some food bacterial pathogens such as *E. coli*(Jothikumar and Griffiths, 2002), *M. paratuberculosis*(O'Mahony and Hill, 2002), *S. enteritidis*, *S.typhimurium*, *Sh.sonnei*, *Sh.flexneri*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *C. jejuni*, *V.cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *A. hydrophila*, *A. trota*, *S. aureus*, *C. perfringens*, *B. cereus*(Fukushima et al., 2003), *L. monocytogenes*(Jothikumar et al., 2003), *E.*sakazakii(Liu et al., 2006), *C.botulinum*(Fenicia et al., 2007), *P.shigelloides*(Gu and Levin, 2008) and *K. pneumonia*(Sun et al., 2010) were used for their specific detection in designed SYBR green real time PCRs.

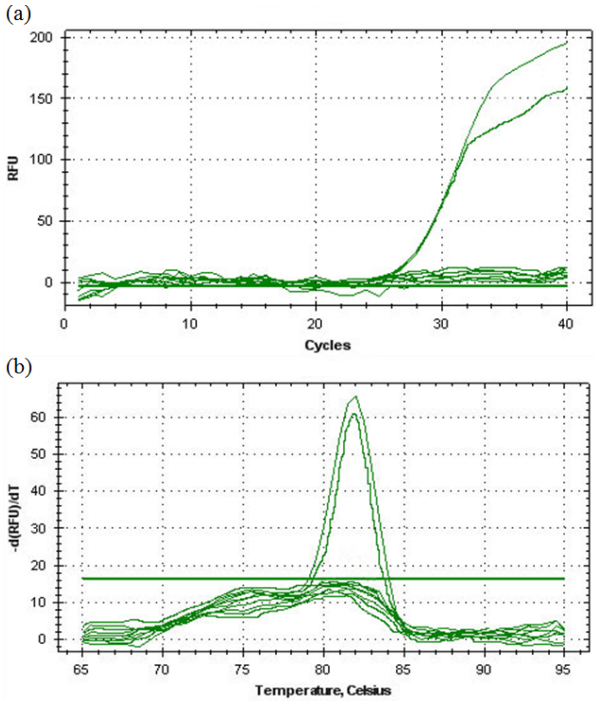

Figure 3. (a) Real time PCR amplification of *B. subtilis*aprE gene. (b) Melting curve analysis of SYBR green real time PCR product of *B. subtilis*(in broth culture as positive control and induced contaminated dough) after 40 cycles in presence of negative control bacterial species used for conventional PCR. The melting temperature is calculated as 82.2 oC. No non-specific peaks are present.

Generation of calibration curves

Identity of the cloned insert was confirmed by direct sequencing using the universal sequencing primer M13F and then reference standards of plasmid DNA were constructed. With these standards, real time PCR assay can be used to directly detect or accurately quantify the dough samples containing *B. subtilis*, which is usually the main pathogen in rope spoilage and also a potential source for foodborne illnesses. Fig. 4 (a) and Fig. 5 (a), show the typical amplification plot (ΔRn or RFU versus PCR cycle) constructed from fluorescence data generated from DNAs corresponding to 2×10^1–2×10^8 copies per ml or g, respectively for reference standards of cloned insert and *B. subtilis* cells in artificially contaminated dough.

In a real time PCR, fluorescence of reaction mixtures just before the denaturation step of each amplification cycle, and the cycle number at which fluorescence crosses a specific threshold value in the exponential phase of amplification are being monitored (designated as C_T or the threshold cycle). The C_T is thus a measure of the quantity of

transcript of interest, and target DNA copy number and C_T values are inversely related. The significant portion of each curve is the place along the C_T axis where each curve departs from the background. The angle and linearity of each curve is related to the efficiency of the PCR reaction with straight and nearly vertical slopes representing more nearly optimal PCR conditions (Bustin et al., 2009; Dhanasekaran et al., 2010). The calibration curve plotting C_T values against known serial dilutions of *B. subtilis* DNA, ranging from 2×10^1 to 2×10^8 copies per ml or g of samples is shown in Fig. 5 (b). The linear range of this real time PCR assay was 2×10^8 to 2×10^2 copies/ml with the best fit regression equation y= -3.0357x + 37.107, (R^2= 0.9993) for standard references of cloned insert. Standard curve also showed linear relationship between C_T and log CFU for serially 10 fold diluted *B. subtilis* cells in artificially contaminated dough with linear regression coefficient of R^2= 0.9887, (y= -3.1429x + 39.119). These results indicate that the reference standards were suitable for quantitative assay (Bustin et al., 2009; Martínez et al., 2011).

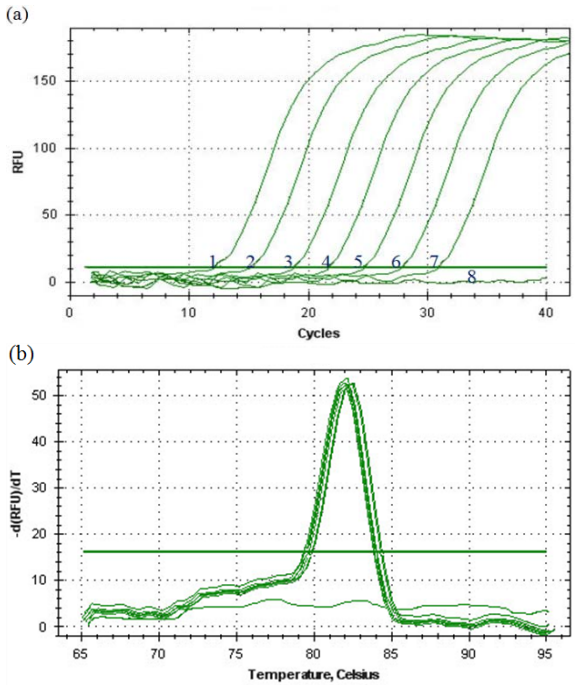

Figure 4. (a) The amplification profile of real time PCR of reference standards. The concentrations of cloning vector ranging from 2×10^8 to 2×10^1 copies/ml: (1) 2×10^8, (2) 2×10^7, (3) 2×10^6, (4) 2×10^5, (5) 2×10^4, (6) 2×10^3, (7) 2×10^2 and (8) 2×10^1. (b) The melting curve of amplification products.

Practical sensitivity of the real time PCR assay

The real time PCR assay was evaluated for practical sensitivity using artificially contaminated dough samples, as described before. The six inoculated samples ranging from 2×10^8 to 2×10^3 number of inoculated cells (CFUs), showed positive fluorescence signals with different C_T values (the last positive fluorescence signal was obtained at a C_T value of 29.5 cycles) and the expected melting temperature, was constant at approximately 82.2 ^oC, while one non-inoculated sample was tested negative by this method (Fig. 5 a). The detection limits of *B. subtilis* in standard references of cloned insert and artificially contaminated bread dough respectively, were determined as 200 CFU/ml and 2000 CFU/g, using SYBR green real time PCR and without the need for further enrichment steps.

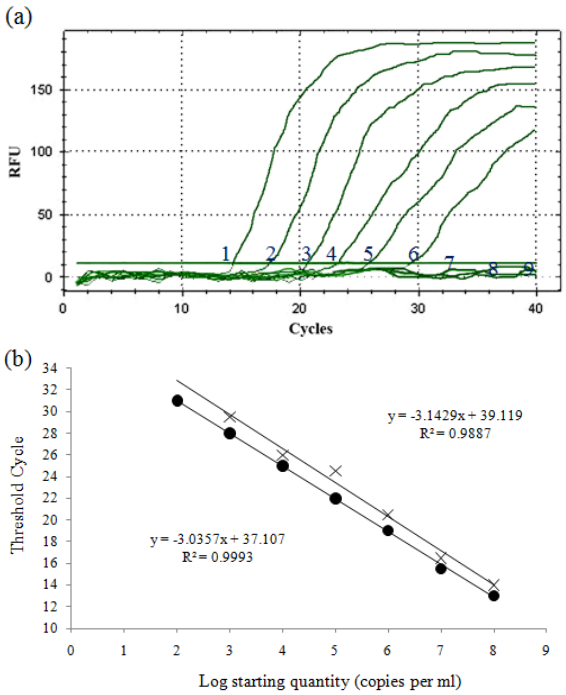

Figure 5. (a) Detection limit in model samples by SYBR green real time PCR assay without enrichment. The amplification profile of 10 fold serial dilutions of *B. subtilis*cells in artificially contaminated dough samples. The concentrations of bacteria inoculated in samples ranging from 2×108 to 2×101 CFU/g: (1) 2×108, (2) 2×107, (3) 2×106, (4) 2×105, (5) 2×104, (6) 2×103, (7) 2×102, (8) 2×101 and (9) negative control (no bacteria). (b) Standard curves as generated from threshold cycle (CT) numbers of a 10 fold dilution series of the new constructed reference standards (2×101–2×108 copies/ml). Standard curves showing the linear relationship between CT and log CFU *B. subtilis*cells in artificially contaminated dough (×) and reference standards of cloned insert (●).

Discussion

Molecular methods, such as conventional PCR and real time PCR, have been used to detect or quantify specific pathogens in food samples. In our procedure, a SYBR green real time PCR was developed with new primer pairs for detection and quantification of *B. subtilis* in dough used for bread making. Specificity of the reaction was confirmed

by melting temperature analysis, which was constant for the amplicon obtained (T_m of 82.2 ^oC). The linear range of this real time PCR assay was 2×10^8 to 2×10^2 copies/ml for standard references of cloned insert. Standard curve also showed the linear relationship between C_T and log CFU for serially diluted *B. subtilis* cells in artificially contaminated dough. This assay enabled us to differentiate *B. subtilis* from main related and unrelated bacterial species that may be naturally present in dough. The assay could detect approximately 2000 CFU/g dough without enrichment and showed potential for quantifying this bacterium with the new constructed reference standards. Since the typical cell counts of *B. subtilis*in rope spoilage have been reported about 10^5 CFU/g of dough (Rosenkvist and Hansen, 1995; Sorokulova et al., 2003), the samples can be processed and analyzed by this procedure, for detecting and quantification of mentioned pathogen in less than 3 h.

In recent SYBR green real time PCR studies for detection of *Bacillus* spp., in food samples, Fricker*et al.*(Fricker et al., 2007) showed that their developed assay, depending on the direct DNA isolation method is suitable for detection of 10^1 to 10^3 CFU/g emetic *B. cereus* in food samples without enrichment. Martínez-Blanch *et al.*(Martínez-Blanch et al., 2009)constructed calibration curves for different food matrices, and it had a wide quantification range of 6 log units using both serial dilutions of purified DNA and calibrated cell suspensions of *B. cereus*. The detection limit of that assay in artificially contaminated liquid egg and reconstituted infant formula was about 60 CFU/ml of food samples. The study of Wehrle*et al.*(Wehrle et al., 2010) showed that artificial contamination of three different food matrices including baby food cereal, rice pudding and carrot puree with distinct bacterial counts revealed a detection limit of 10^1 CFU/g enteropathogenic *B. cereus* cells after overnight enrichment. Although most real time PCR assays in themselves are characterized by high precision and reproducibility, the accuracy of the obtained data is largely depended on several other factors such as sample preparation and quality of the standards. The obtained absolute numbers are always calculated relative to the standard (RNA, cDNA, plasmid DNA, genomic DNA) and largely depend on the accuracy of used standard. Due to the lack of standardized reference material, the accuracy of the obtained data has to be checked during the establishment of the assay by comparison with other established assays. This is the major task for molecular diagnostics using real time PCR system (Dhanasekaran et al., 2010; Kennedy and Oswald, 2011; Martínez et al., 2011; Ponchel et al., 2003). As mentioned, reliable standards are essential for qPCR and it also has been found that the copy numbers vary (due to degradation of standards) over the period of time during storage at 4 °C and −20 °C, which affects PCR efficiency significantly. Based on the results obtained by Dhanasekaran*et al.*(Dhanasekaran et al., 2010) the cloned target sequences are noticeably more stable than PCR products. This could lead to substantial variance in results using standards constructed by different routes, and therefore, cloned insert was used for standard preparation in this study.

A number of fluorescent probe based real time PCR studies have been carried out to detect *Bacillus* spp., in foods or other samples using TaqMan probes or molecular beacons (Fernández-No et al., 2011; Fricker et al., 2007; Gore et al., 2003; Martínez-Blanch et al., 2009). These fluorescent probe based assays require availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied. Use of the double stranded DNA binding dye, SYBR green, for detection of PCR products has overcome this limitation by allowing real time PCR to be applied without the need for probes linked to fluorescent molecules. Protocols that are already in use for classic PCR can thus be used with only slight modifications (Aarts et al., 2001; Gibson et al., 1996). In the absence of probes, specificity of the reaction is determined by melting temperature of the amplicon obtained. SYBR green lacks the specificity of fluorescent DNA probes but has the advantage of allowing a DNA melting curve to be generated and software calculation of the $T_m$of the amplicon after the PCR. This allows identification of the amplified product and its differentiation from primer dimers, which also result in a fluorescent signal with SYBR green but usually have a lower $T_m$value. The fluorescent signal is measured immediately after the extension step of each cycle because thermal denaturation yielding single stranded DNA eliminates fluorescence. A software plot of the negative first derivative of the thermal denaturation plot yields a bell shaped symmetrical curve, the midpoint of which corresponds to the $T_m$value for the amplified product. Interference of the amplicon's signal by the signal resulting from primer dimer formation can be eliminated by raising the temperature to a critical point that is above the $T_m$of the primer dimer formed (resulting in thermal denaturation of the primer dimers) but below the $T_m$of the amplicons prior to measuring the intensity of fluorescence emission (Levin, 2010; Mackay, 2004).

In **conclusion**, this SYBR green real time PCR assay provides a rapid, highly sensitive tool for the detection of *B. subtilis*and quantification of the bacterium cells in a complex food matrix after suitable sample preparation. With further validation, this assay could be used by regulatory and food industry laboratories to rapidly screen for *B. subtilis*contaminations. Considering this bacterium association with foodborne illnesses and also its thermal resistance, especially to commercial baking conditions and spore forming ability indicates the importance of this assay. In contrast to the culture dependent methods, it offers significantly higher accuracy and speed, which are crucial criteria when comes to food safety and high volume of referred samples.

Acknowledgment

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