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LIGHT SCATTERING SENSOR FOR RAPID DETECTION OF *BACILLUS* CEREUS IN CHICKEN MEATS

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ABSTRACT

Bacillus cereus is an opportunistic pathogen causing food poisoning, manifested by diarrhea or emetic illnesses. BARDOT (BActerial Rapid Detection using Optical scattering Technology) directly capturing phenotypic characteristics of colonies following standard agar plating, providing non-destructive, high-throughput analysis and real-time detection of colonies on agar plate without any labeling reagents or probes. This study implement light scattering sensor in detection and identification of B. cereus on genus level in mixed cultures and in artificially inoculated chicken samples using PRM (phenol red mannitol) agar. Software analysis and PCR confirmation showed that BARDOT successfully detect 100% of B. cereus in mixed culture and >90% of artificially inoculated chicken samples. This results demonstrates that BARDOT could be used as a screening tool to identify Bacillus cereus from other pathogens and background flora on PRM agar.

Keywords: Bacillus Cereus, light scattering sensor, BARDOT and Chicken.

INTRODUCTION

B. cereus is a Gram-positive, spore-forming, flagellated, rod-shaped bacterium. Because of its ubiquitous nature, *B. cereus* is widespread in nature and commonly found in air, soil and water **(Vilain et al., 2006).** It can easily be spread to the foods of plant origin and through cross contamination to other foods such as milk, meat and meat products **(Granum 1994 and Larsen and Jorgensen, 1997).** Nowadays, meat and meat products are the most important incriminated food in *B. cereus* food poisoning as contamination and multiplication of *B. cereus* organisms in raw meat and their products is of major concern as public health hazard. *B. cereus* Spores are resistant to ecological stressors more than the vegetative form due to their metabolic dormancy and substantial nature **(Jenson and Moir, 2003).**

B. cereus is the etiologic agent of two types of food associated illnesses emetic and diarrhoeal type. The emetic illness is a food intoxication resulted from ingestion of a cyclic peptide toxin called cereulide formed during growth of *B. cereus* organisms in food; this form is characterized by a short incubation period and recovery time. The signs appears 2-5 h following ingestion of contaminated food includes nausea, vomiting and abdominal cramping with recovery usually within 6–24 hours **(Schoeni and Wong, 2005 and Senesi and Ghelardi, 2010)**.

The diarrhoeal illness is caused by enterotoxins produced by *B. cereus* growth inside the host small intestine. The incubation

period is 8–16 hours and the illness usually lasts within 12–14 hours, however it might continue for several days. Signs are usually mild with abdominal cramps, watery diarrhoea and nausea **(Granum, 2007)**. Few cases recorded both types of toxin are produced (Montville and Matthews 2005). However neither of illness is considered life threatening illness **(Jenson and Moir, 2003)**. *B. cereus* food poisoning occur as the spore survive the cooking temperature or as a result of poor temperature control during the processing and holding of the food resulting in bacterial vegetation and multiplication. Ingestion of food contaminated with 10^5-10^6 *B. cereus*/g resulted in food poisoning illness **(Kramer and Gilbert 1989)**.The incidence of *B. cereus* has been reported in meat and meat products **(Bachhil and Negi, 1984; Bachhil and Jaiswal, 1988; Willayat et al., 2007; Hafiz et al., 2012 and Rao et al., 2012)**. Food poisonings cases resulted from *B. cereus* in different countries is not a reportable illness and is not always diagnosed **(Kotiranta et al., 2000)**.

Detection and identification of *B. cereus* from food samples generally based on three chief methods basically, first: the traditional culture method based on agar medium, colony morphology and further biochemical confirmation. Secound: immunoassays using specific antibodies targeting either the cells or toxins **(Chen and Ding, 2004; Dietrich et al., 2005 and Moravek et al., 2006)**, and third is molecular techniques as PCR that identifies toxin production genes **(Fricker et al., 2007; Martinez-Blanch et al., 2009 and Wehrle et al., 2009)**. However, many limitation facing these methods leading to false negative results due to matrix associated inhibitors and also may not differentiate living from dead bacteria **(Hedman and Radstrom, 2013)**, and do not allow injured cell recovery due to lack of enrichment step.

Therefore, continuous looking for an alternative techniques, especially those that combine the advantage of recovery of the target pathogen in addition to being highly specific, rapid, and sensitive is a major challenging task for regulatory agencies and food industries to control food borne pathogens. Currently optical biosensors are of increasing interest as they provide fast, non-destructive, sensitive, and specific results **(Bhunia, 2011, 2014; Homola, 2008 and Sharma and Mutharasan, 2013)**. Light scattering BARDOT greatly offer a high throughput screening tools to test food samples in competitive time compared to other conventional methods and without the need for labeling reagents **(Bhunia, 2014)**.

Therefore, the aim of this study was to investigate implementation of BARDOT optical sensors for specific and accurate detection of *B. cereus* in mixed cultures and in artificially inoculated chicken samples.

MATERIALS AND METHODS

2.1. Bacterial cultures and growth media

B. cereus and non-Bacillus cultures (*Escherichia coli* O157:H7, *Salmonella. Typhimurium*, *Salmonella. Enteritidis* PT21, *Citrobacter freundii* and *Hafnia alvei*) were used in the experiments. All cultures were grown from 80- °C frozen glycerol stocks by inoculation into brain heart infusion broth (BHI, Becton Dickinson (BD), flowed by overnight incubation at 37 °C in a shaker incubator. To develop a light-scatter image library, selective agar phenol red mannitol (PRM; BD) dehydrated media were prepared as the manufacturer's instruction.

2.2. Light scattering sensor based detection

All laboratory cultures were overnight grown in BHI broth, serially diluted in 20 mM phosphate buffered saline (PBS), pH 7.4, plated onto phenol red mannitol agar (PRM) and incubated at 37 °C for 7:8 h or to achieve a colony diameter of about 1 mm (0.8 ± 0.2 mm), measured under a phase-contrast microscope (Leica,Wetzlar, Germany) Leica Application Suite version 4.2.0 (Leica Microsystems, Switzerland). Plates were screened using BARDOT machine **(Figure 1**), reported previously **(Banada et al., 2009 and Singh et al., 2015)** and the colony scatter patterns were matched against the Bacillus- non Bacillus scatter image libraries generated in our previous study (**Singh et al., 2015)**. and the results were presented as positive predictive values percent (PPV%) **(Singh et al., 2014)**.

2.3.BARDOT instrumentation and image analysis.

BARDOT (bacterial rapid detection using optical scattering technology) is a laser based forward light scattering sensor that utilizes a 635-nm red diode laser beam to capture scatter signatures of individual ~1-mm diameter colonies for real-time interrogation on the plate (**Bhunia, 2011)**. The method is used for direct capturing of phenotypic characteristics of bacterial colonies obtained by classical microbiological methods to provide non-destructive, high throughput analysis and real-time detection on agar plates without any labeling reagents or probes **(Banada et al., 2009)**.

2.4. Detection and identification of *B. cereus* in Mixed cultures.

Overnight grown *B. cereus* were mixed in equal amount individually and collectively with each of *Citrobacter freundii*, *E. coli* O15:7H7 or *S. Enteritidis* PT21 and *Hafnia alvei* then 100 ul aliquot was serially diluted in PBS and surface plated on PRM agar till the colony size of 1mm for BARDOT analysis and The colony scatter patterns matched against Bacillus and non Bacillus scatter image libraries using DOTBAR software with further PCR confirmation for the BARDOT identified colonies.

2.5.Detection and identification of *B. cereus* from artificially inoculated food samples.

Chicken breast samples were purchased from local grocery stores in West Lafayette, Indiana and 30 g of chicken samples were artificially inoculated with 100 µl overnight grown *B. cereus* ATCC14579, placed in a biosafety cabinet for 24h. Samples were transferred into stomacher bags (Seward, Cincinnati, OH, USA), resuspended in 270 ml of 20 mM PBS-T (0.025%). Samples stomached, homogenates were serially diluted in PBS and 100ul plated in duplicate on PRM agar Plates Incubated at 37°C for 7h, Plates are scanned with BARDOT and scattered pattern analyzed using DOTBAR software, Colonies were picked based on BARDOT identification for PCR confirmation. Chicken samples without inoculation were used as negative controls.

2.6. DNA extraction and PCR analysis.

DNA was extracted using the boiling method **(Ngamwongsatit et al., 2008)** PCR was performed using *B. cereus* gyrB gene specific primers (BcF: 5′ GTTTCTGGTGGTTTACATGG-3′;BcR: 5′-TTTTGAGCGATTTAAATGC-3′) for the forward and reverse primer respectively **(Kuo and Chak, 1996 and Manzano et al., 2003)**. The PCR reaction mixture (25 µl) contained 200 µM of each dNTP, 2.5 mM of MgCl2, 1×

GoTaq Flexi buffer, 1 U of GoTaq Flexi DNA polymerase (Promega), 0.2–0.3 µM of primers, and 60– 90 ng of template DNA. PCR reactions were performed using the following cycling conditions: initial denaturation at 94 °C for 2 min followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 54 °C for 2 min, and elongation at 72 °C for 2 min. Ultrapure sterile water was used as a negative control. aliquots were analyzed by electrophoresis in 1.5 % agarose gel containing 1 ml of ethidium bromide solution and visualized under UV light.

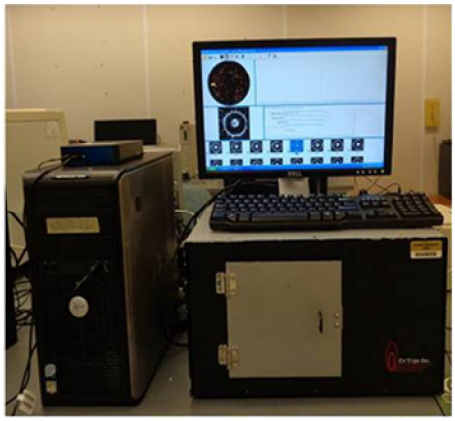

Figure 1: Photograph of a laser optical sensor,
BARDOT (Bacterial Rapid Detection using Optical scatter Technology)

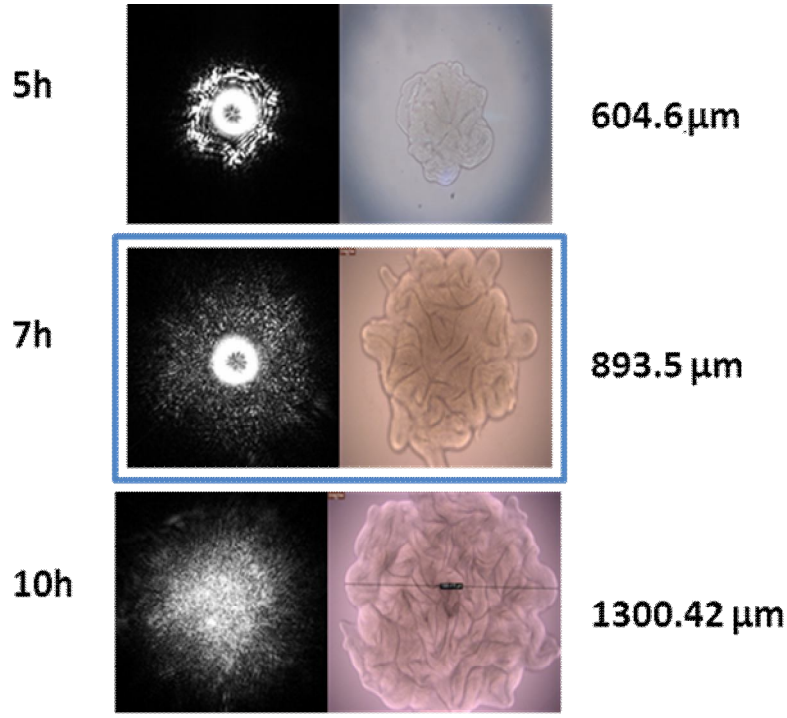

Figure 2: Colony images and scatter patterns of B. cereus on PRM agar at different time points.

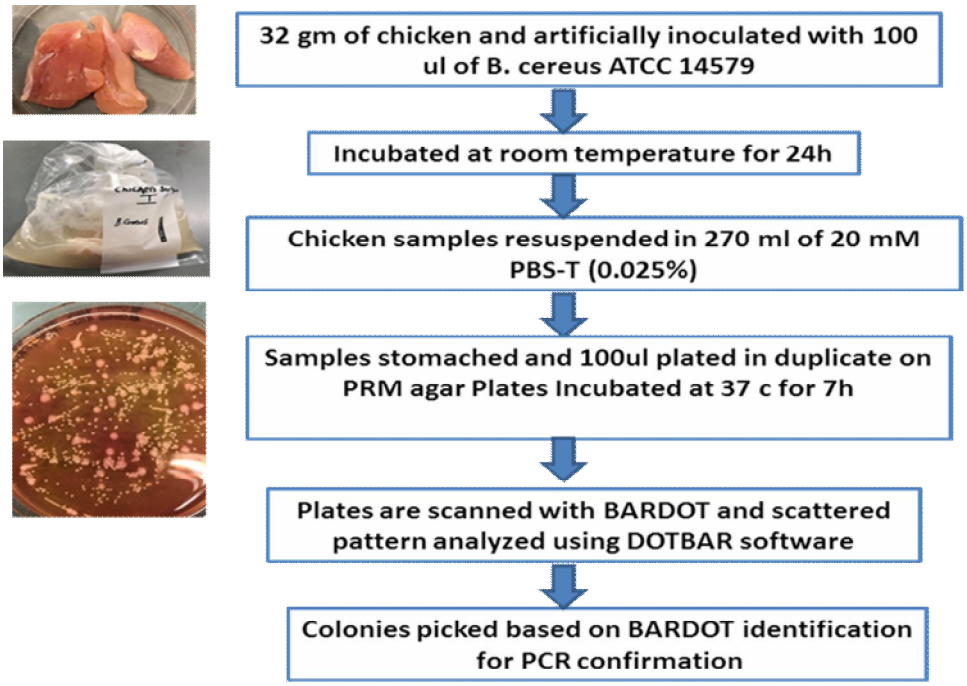

Figure 3: Flow diagram showing the procedure for the detection of B. cereus in chicken samples using optical scatter biosensors

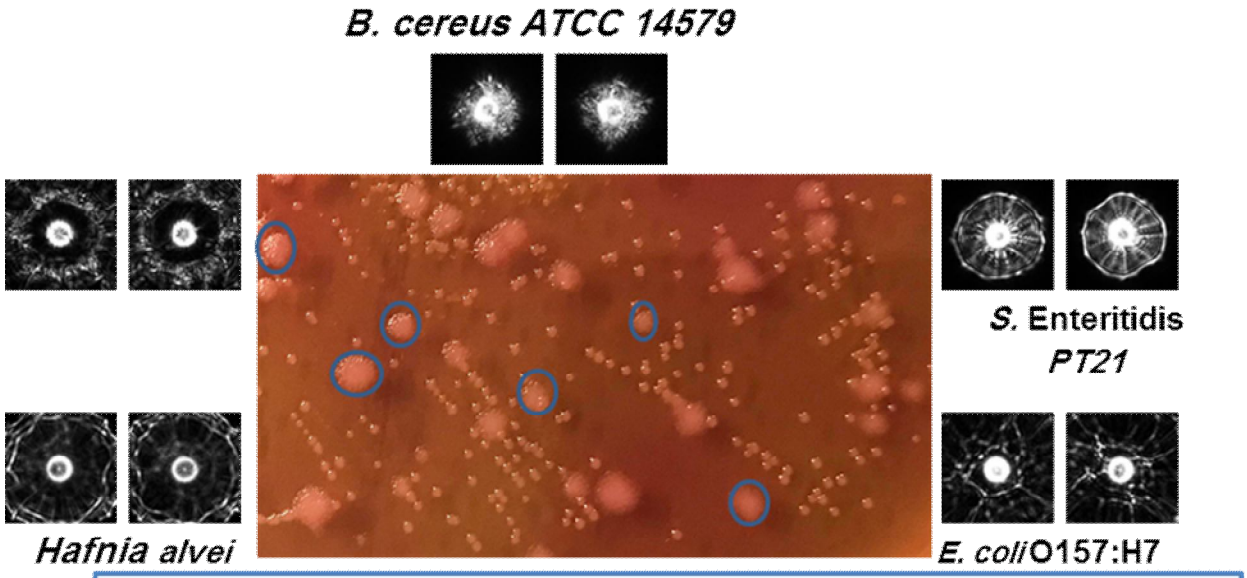

Figure 4: Representative mixed PRM agar plate and BARDOT scatted pattern. The marked colonies are B. cereus , other colonies are either *E.* Coli O157:H7 or *S.* Enteritidis PT21

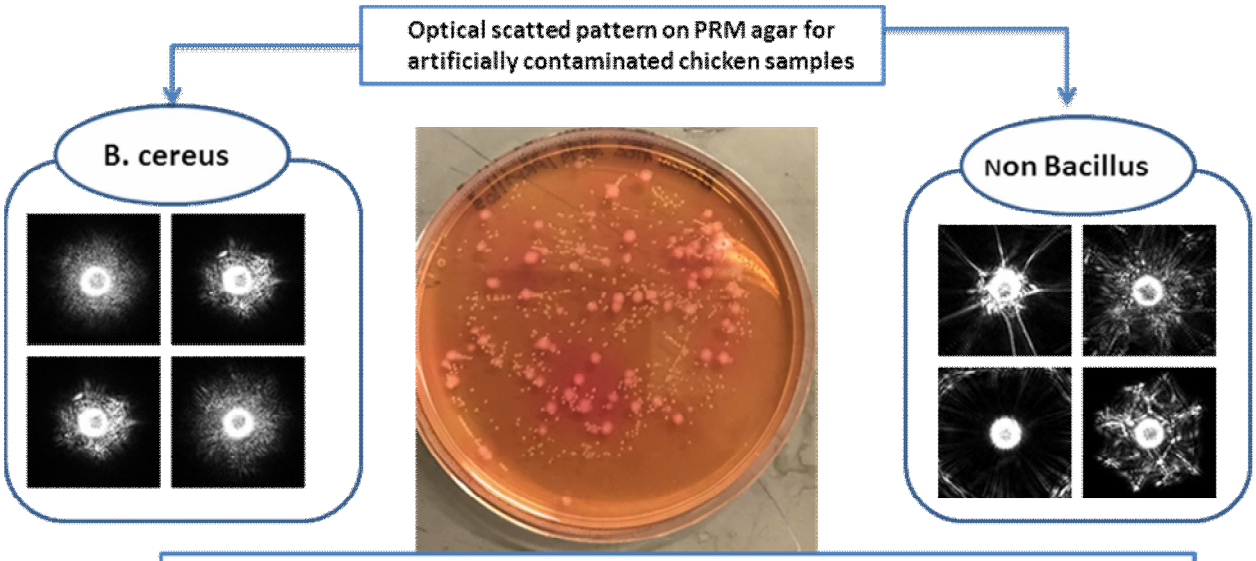

Figure 5: a-artificially contaminated chicken samples on PRM agar b-Scattered pattern of B. Cereus From artificially contaminated chicken samples. C-Scattered pattern of natural isolates from chickens

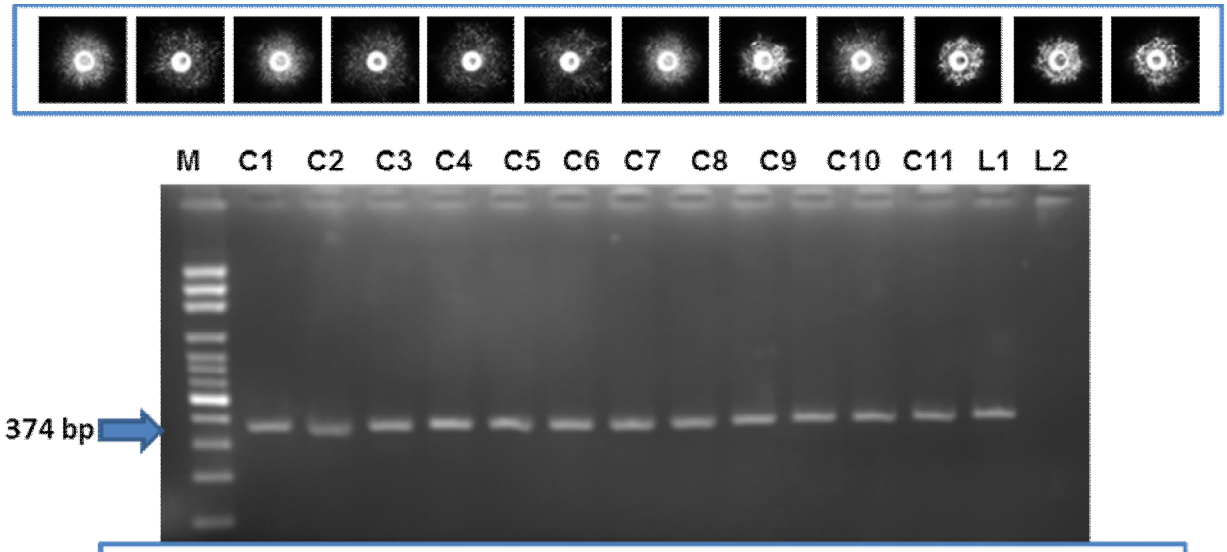

Figure 6: Representative agarose gel C1:C12 showing PCR confirmation of BARDOT identified colonies on PRM agar using B. cereus gyrB gene specific primers (374 bp). M: 100 pb ladder, L1: pure culture positive control, L2: negative control.

RESULTS AND DISCUSSION

Rapid and sensitive detection techniques are essential for detection and control of foodborne pathogens in food processing facilities. The conventional culture based methods provide reliable results but the analysis time, labor and the cost associated with these methods often pose a great inconvenience to industrial applications. Biosensor based detection methods are considered to be promising emerging technologies which are capable of fulfilling the current needs for providing results rapidly with improved sensitivity and specificity **(Bhunia 2008 and 2011).**

3.1. BARDOT-based detection.

For optical scattered sensor, it is essential to select an agar medium capable of yielding well differentiating scatter patterns for *B. cereus* from other foodborne pathogen and contaminants . PRM agar generated differential scatter patterns with high PPV% on genus level using Bacillus -non Bacillus library created in our previous study, in addition to the newly added classes including (*Escherichia* coli O157:H7, *Salmonella* enterica serovar Typhimurium, *Citrobacter freundii* and *Hafnia alvei*). The Bacillus- non Bacillus scatter image library helps detect *B. cereus* at the genus level with high accuracy (PPV 95%); this results comes in agreement with results obtained by **(Singh et al., 2015)**. who stated that PRM agar showed high classification accuracy on genus level for Bacillus and low PPV% on species level between Bacillus due to overlapping scattered pattern. also as reported earlier for other foodborne pathogens **(Banada et al., 2009; Huff et al., 2012; Singh et al., 2014; Tang et al., 2014 and Abdelhaseib et al., 2016)**. The newly added pathogens are highly distinguishable pattern on PRM agar. However they develop the 1 mm characteristic colony in relatively longer time (11-15h) compared 7h required by *B. cereus* to develop its pattern. this result provides time elapsed differentiation of Bacillus versus non Bacillus pattern.

3.2. Time-resolved scatter patterns of colonies of *B. cereus* versus non Bacillus

Time elapsed measurement for the colony diameter showed that *B. cereus* require 7 h to reach the targeted size to create distinguishable scattered pattern, that matched (**Singh et al., 2015)**.

on the other hand other pathogens and contaminant require 11-15h to develop 1 mm diameter colony and scattered pattern. As between 7-8h *B. cereus* developed well-defined scattered pattern when the colony size measures (0.8± 0.2 mm), 5-6 h after incubation the pattern still raw and small in size. ten hours later scatter pattern become grainy and majority of the pattern features were missed as the colony size reach > 1.2 mm **(Figure 2).**

3.3.Bacillus and non-Bacillus scatter patterns

In this section we compare the pattern created by *B. cereus* colonies on PRM agar in addition to the newly added group, Results showed that *B. cereus* creating irregular shaped small grainy pattern with outstanding spokes and lack circular ring, this pattern reflect the non-symmetrical profile, and rough colony surfaces of *B. cereus*. This scatter patterns were highly distinguishable as on the other hand all non-Bacillus cultures tested in this study were circular, symmetrical with smooth surface texture and their scattered patterns showed typical concentric rings with both *S.*

Enteritidis PT21, *S. Typhimurium* and with outward spokes for *Citrobacter freundii, E. coli* O15:7H7 EDL 933 and *Hafnia alvei* . The colony scatter pattern mainly depend on genotypic and phenotypic characteristics of the colony, including but not limited to shape, consistency, and surface texture, agar media used, and the time needed to reach 1mm diameter colony **(Banada et al., 2009; Bae et al., 2011; Huff et al., 2012 and Singh et al., 2014)**.

3.4. detection of *B. cereus* mixed cultures.

BARDOT was successful in detecting and differentiating colonies of *B. cereus* from mixed cultures with either two pathogens or multipathogen culture mix based on scattered signature.

Total of 20 colonies ware picked up from all the culture cocktails and further confirmed using PCR 100% of the tested colonies were *B. cereus*. In details 5 colonies from each mixture (*B. cereus- S. Enteritidis* PT21 mix), (*B. cereus- E. coli* O15:7H7 EDL 933 mix), (*B. cereus- Citrobacter freundii - Hafnia alvei* mix) and finally (*B. cereus -Citrobacter freundii-E. coli* O15:7H7 EDL 933- *S. Enteritidis* PT21-*Hafnia alvei* mix*)*. Time required to develop the scattered pattern was also supporting factor to differentiate the *B. cereus.* **(Figure 4)**

3.5.Artificially inoculated chicken samples.

We artificially inoculated raw chicken with *B. cereus* to simulate natural contamination, and tested the efficacy of the optical sensors to detect and differentiate *B. cereus* scattered pattern in presence of natural contamination of raw chicken. DOTBAR classification with accurately (>98%) identified as *B. cereus*. validation of the light scattering sensor and software analysis by PCR using *B. cereus* specific primers. In total, 20 colonies identified with BARDOT (representative images, **Figure 5b**) from chicken, 19 out of 20 (95%) were confirmed by PCR as B. cereus. **(Figure 5,6)** However, this study demonstrates the application of light scattering sensor to detect *B. cereus* based on the signature scatter pattern and thus could be used as a rapid screening tool, which can produce results within 24 h starting with the food or environmental samples.

The laser optical sensor described in this section was developed as a real time, label free, non-destructive, on plate detection and identification tool for use with bacterial pathogens **(Banada et al., 2007; Banada et al., 2009; Bhunia, 2011 and Huff et al., 2012).** The main limitation of BARDOT based detection is that it may produce overlapping patterns of target organisms with other patterns of background flora. However, when the scatter image library was used, it produced differential identification and a cut-off value below 80% was considered negative. Furthermore, cross validation of images for presumptive positive colonies with the scatter image library for potential background bacteria on that specific agar plate would also help improve data analysis and interpretation of results. In addition, continuous improvement in scatter patterns image library with more strains will also improve BARDOT based detection of bacteria from naturally contaminated samples.

Conclusion

The present work highlights the application of light scattering sensors to improve food safety through quick screening of food samples. The novelty of label-free on plate colony screening technology, BARDOT sensors could help screening of food products for the presence of *B. cereus* on genus level in food manufacturing, and public health laboratories. Based on the BARDOT screening results, the target pathogens can be further confirmation, moreover it provides useful

tracking and epidemiological survey through molecular techniques, genome sequencing, and effectively respond different sanitization protocols.

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الملخص العربي

مجسات التشتت الضوئي للكشف السريع عن جراثيم باسيلس سيريس في لحم الدجاج

مها عبد الحسيب ، ارون بونيا

تعد باسيلس سيريس جراثيم انتهازية تسبب التسمم الغذائي محدثه الإسهال أو القي وتقوم مجسات التشتت الضوئي بالتقاط الخصائص المظهرية للمستعمرات مباشرة بعد زراعتها علي بيئه الاجار وتقدم تحليلات عالية الإنتاجية وسريعه الكشف و غير مدمرة المستعمرات على بيئه الاجار دون الحاجه لاي كواشف ,استخدمت هذه الدراسة مجسات التشتت الضوئي في الكشف عن باسيلس سيريس في العينات المختلطة وفي عينات الدجاج الملوثه اصطناعيا وأظهر تحليل النتائج ان مجسات التشتت الضوئي كشفت بنجاح عن ١٠٠٪من باسيلس سيريس في العينات المختلطة واكثر من ٩٠٪ من عينات الدجاج الملوثه اصطناعيا. هذه النتائج تبين أن مجسات التشتت الضوئي يمكن أن تستخدم كأداة فحص لتحديد باسيلس سيريس من مسببات الأمراض الأخري على بيئه الفينول مانيتول الأحمر.