

## A rapid qualitative assay for detection of *Clostridium perfringens* in canned food products\*

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*Clostridium perfringens* (MTCC 1349) is a Gram-positive, anaerobic, endospore forming, and rod-shaped bacterium. This bacterium produces a variety of toxins under strict anaerobic environment. *C. perfringens* can grow at temperatures ranging between 20°C and 50°C. It is the major causative agent for gas gangrene, cellulitis, septicemia, necrotic enteritis and food poisoning, which are common toxin induced conditions noted in human and animals. *C. perfringens* can produce produce four major types of toxins that are used for the classification of strains, classified under type A–E. Across the globe many countries, including the United States, are affected by *C. perfringens* food poisonings where it is ranked as one of the most common causes of food borne infections. To date, no direct one step assay for the detection of *C. perfringens* has been developed and only few methods are known for accurate detection of *C. perfringens*. Long detection and incubation time is the major consideration of these reporter assays. The present study proposes a rapid and reliable colorimetric assay for the detection of *C. perfringens*. In principle, this assay detects the para nitrophenyl (yellow colour end product) liberated due to the hydrolysis of paranitrophenyl phosphatidyl choline (PNPC) through phospholipase C (lecithinase). Constitutive secretion of phospholipase C is a characteristic feature of *C. perfringens*. This assay detects the presence of the extracellular lecithinase through the PNPC impregnated impregnated probe. The probe is impregnated with paranitrophenyl phosphatidyl choline ester, which is colourless substrate used by lecithinase. The designed assay is specific towards PNPC and detects very small quantities of lecithinase under conditions used. The reaction is substrate specific, no cross reaction was observed upon incubation with other substrates. In addition, this assay gave negative results with other clostridium strains, no cross reactions were observed with other experimental strains like *C. tetani*, *C. botulinum*, *C. acetobutyricum*, *Bacillus subtilis*, and *Escherichia coli*. This assay is extremely rapid and provides reliable and reproducible results within one hour of incubation at 37°C.

**Key words:** *Clostridium perfringens*; lecithinase; paranitrophenyl phosphatidyl choline ester

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**Abbreviations:** cm, centimetre; h, hour; RPM, revolutions per minute; CFU, colony forming unit; nm, nanometre

### INTRODUCTION

Canning is generally regarded as a safe method for preserving food over prolonged periods of time, if practiced properly. In the canning process, foodstuff kept in jars or similar containers is heated above the boiling temperature of water. This process kills the microbes without compromising the quality of the foodstuff. During this heating process, the air is driven out of the jar and as it cools a vacuum seal is formed. This vacuum seal prevents the air from getting back into the product and bringing with it possible contaminating microorganisms. Canning efficiently removes microbes. Occasionally, few anaerobic microbes and spores of anaerobes that survive under this harsh killing procedure may contaminate the food products.

*Clostridium perfringens* is a Gram-positive anaerobic spore-forming bacterium that causes life-threatening gas gangrene and mild enterotoxaemia in humans, although it colonizes humans and animals as a normal intestinal flora. The organism is known to produce a variety of toxins and enzymes that are responsible for the severe myonecrotic lesions. Among five types (A to E), *C. perfringens* type A is known to cause two types of infectious diseases in humans. The first is gas gangrene (Clostridial myonecrosis), in which *C. perfringens* typically contaminates a skin wound as a spore from the soil and then rapidly grows and produces various histolytic toxins and enzymes that destroy the surrounding muscles and connective tissues. The second type of common disease is food poisoning caused through the few strains of Clostridia (approximately 5% of all *C. perfringens* type A isolates) of *C. perfringens* producing enterotoxin (CPE) (Ohtani *et al.*, 2013). The CPE-producing strains are responsible for nearly one million cases of *C. perfringens* food poisonings annually. It is also ranked as the second most commonly reported bacterial food-borne disease in the United States (Jihong Li *et al.*, 2013). When food products contaminated with vegetative CPE-producing *C. perfringens* cells are ingested, the microbes colonize the intestine and begin to sporulate within 24-h of incubation. During the sporulation process, a large amount of CPE is produced. CPE forms pores in the membranes of intestinal epithelial cells and causes watery diarrhoea.

Presently, the reported bioassays detect clostridia through culturing on specific microbiological media. This culturing method requires a long time for detection and it also requires an extra effort to create oxygen free environment for growth. Countries in the developing world, however, require a rapid, inexpensive and simple method to perform tests that do not require a highly sophisticated laboratory and equipment. To address

Table 1. Microbial cultures used for this study

Name	Characteristics	Optimum growth conditions	Reference
<i>Clostridium perfringens</i> (MTCC 450)	Lecithinase <sup>+</sup>	Temperature 30°C, Reinforced Clostridial Agar, In Anaerobic jar equipped with Anaero-gas pack	This work
<i>Clostridium botulinum</i> (ATCC 3502)	Botulin <sup>+</sup> , Lipase <sup>+</sup>	Temperature 37°C, Clostridia agar	(Kirk <i>et al.</i> , 2012)
<i>Clostridium perfringens</i> (MTCC 450)	Lecithinase <sup>-</sup>	Temperature 30°C, Fluid thioglycollate broth	This work
<i>Saccharomyces cerevisiae</i> YJF130	PLC1 (phospholipase <sup>+</sup> )	Temperature 30°C, Glucose yeast extract broth	(Flick & Thorner, 1993)
<i>E. coli</i> DH5a	Wild type	Temperature 37°C, Nutrient broth	-
<i>Bacillus subtilis</i>	Wild type	Temperature 37°C, Nutrient broth	-

the need of developing nations, this study presents a colorimetric paper-based one step assay for the detection of *C. perfringens* in canned food products (Fig. 1). The study further optimizes the assay parameters for rapid detection.

## MATERIALS AND METHODS

**Cultures, media and materials.** This colorimetric biochemical reaction was studied with 6 bacterial species to evaluate the cross reactivity and the specificity of colour reaction. All of the cultures were obtained from Microbial Type Culture Collection Centre (MTCC) Chandigarh, INDIA and American Type Culture Collection Centre, Manassas, USA and are maintained in our laboratory. Culture conditions and their characteristics are listed in Table 1. The cultures were bimonthly transferred into the cultivation medium and stored at 4°C in the course of this study. All of the chemicals were purchased from Sigma-Aldrich. The key substrate, *o*-nitro phenyl phosphatidyl choline, was purchased from Sigma-Aldrich as well.

***o*-nitro phenyl phosphatidylcholine [PNPC] impregnated strip.** Water and ethanol, mixed at an equal ratio, was used for preparation of 1% PNPC solution for loading on an absorbent paper. Absorbent paper (Whatman No.1) was cut into 25 cm long strips, and the width of each strip was fixed at 1.5 cm. Acetone (60%) was used to remove water insoluble impurities attached to the absorbent paper; to remove them, the paper was immersed in the acetone solution and air dried for 2-h at room temperature. An end of the air-dried paper strip

(10 cm long) was fixed to a wooden strip by an adhesive (purchased from a local market). This unfolded strip of paper was completely immersed in 800 microliters of the PNPC solution for nonspecific attachment of PNPC onto the paper surface. The strip was then dried in a hot air oven (EIE instruments) for 1-h and to remove traces of water the strip was further dried in a desiccator for 24-h. A completely dried strip was carefully rolled over the wooden support and stored in a sterile container for further application.

**Substrate specificity of enzyme-substrate reaction; a quantitative estimation.** An enzyme is a biomolecule that is very specific toward its substrate. In order to evaluate the substrate specificity of this developed biochemical assay, six different microbial cultures were incubated in 1% PNPC and 1% Para-nitrophenyl palmitate broth prepared in deionized distilled water. The initial density was adjusted at 100 CFU/ml for the six strains listed in Table 1. This reaction mixture was incubated at 37°C for 1-h, followed by centrifugation (Sigma) at 4000 RPM for 10 minutes. Supernatants were collected from each reaction mix and the absorbance was measured at 450 nm in an UV-Visible spectrophotometer (Systronics). Purified lecithinase and lipase (Sigma-Aldrich) were used as standard controls.

**Two Factor analysis; Microbial load and temperature.** Canned foods are generally stored at a low temperature that ultimately slows down/prevents the growth of *C. perfringens*. Two-factor analysis was performed to evaluate the effect of temperature and microbial load on the designed assay. This analysis was carried out with different microbial load (measured by CFU analysis) in the range of 10<sup>1</sup>-10<sup>11</sup>, at different series of temperatures (2-40°C). To obtain the high-density bacterial biomass, two liters of cultivation broth containing pure bacterial cultures (O.D.=0.8) were subjected to centrifugation. Supernatants were discarded and the pellets were re-suspended in phosphate buffered saline. The concentrated biomass was enumerated through Standard plate count, performed by a serial dilution method (10<sup>8</sup>) of the concentrated biomass. The stock culture for each concentration of microbial load was prepared by this technique. The cultures from the respective stock (10<sup>1</sup>-10<sup>11</sup>) were then incubated at the reaction temperatures for optimization. The

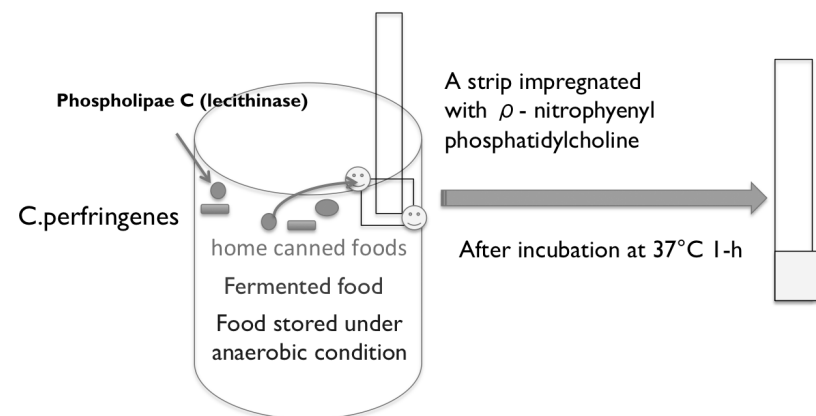


Figure 1. Principles of colorimetric assay

Table 2. Norm of reaction

Microbial load	Incubated in PNPC broth (2%) at 37°C for 1-h	Lecithinase (U/l)	PNPC coated assay strip immersed in culture broth for 10 minutes dried at 37°C for 1-h	Color reaction
10		0.002		
10 <sup>2</sup>		32.3		
10 <sup>3</sup>		42.6		
10 <sup>4</sup>		48.9		
10 <sup>5</sup>		44		
10 <sup>6</sup>		45.8		
10 <sup>7</sup>		52.5		
10 <sup>8</sup>		54.9		
10 <sup>9</sup>		69.9		
10 <sup>10</sup>		92.1		

lecithinase activity was measured after 24-h incubation at different temperatures in the FTB broth.

**Lecithinase assay:** one millilitre of the culture supernatant was incubated in 1% PNPC solution for 1-h; the liberated paranitrophenyl concentration was measured as an absorbance at 450 nm.

**Optimization of assay parameters.** Reproducibility, cost and robustness are desirable features of any diagnostic assay. These parameters were optimized through the series of experiments. Experiments included measurement of critical substrate and enzyme concentration, and the efficiency of colour reaction in the presence of other microbes. The colour reaction was also assayed by incubating the microbes on heterogeneous substrates. The diagnostic range of the assay was determined by experiments that involved the very few clostridia (as few as 10 CFU/ml) and increased to a very high number of Clostridia. Concurrently, in the same experiments, the lecithinase activity was also analysed for different microbial load. The colour intensity was recorded for each assay condition.

**Validation of the mode of lecithinase action through FT-IR.** IR spectra of solutions containing lecithin (a), PNPC (1%) (b), PNPC (1%) + lecithinase (1U) (c) were measured from 4000 cm<sup>-1</sup> to 500 cm<sup>-1</sup> with an FTIR spectrophotometer. An overhead-attenuated total reflection [ATR] accessory was equipped as the sample stage for liquid samples. All spectral measurements were done at 1 cm<sup>-1</sup> resolution. The single beam ATR spectrum from each sample was corrected using a background spectrum of deionized water, and transformed to absorbance units.

## RESULTS AND DISCUSSIONS

### Colour reaction and its specificity assessment

This method of detection of *C. perfringens* is very easy to perform and offers an excellent solution for developing nations. In the presence of *C. perfringens*, the paper strips impregnated with PNPC turn yellow (I). This reaction is very quick, as it gives the positive results just within 1-h of incubation at 37°C. The developed colour

reaction (I) is stable, no change was observed even after 24-h observation (Fig. 2), while the control strip has not shown any colour change in the absence of *C. perfringens*.

A pre-validation test (Table 2) was performed to estimate the assay performance at various microbial loads, as sometimes the canned food products contain only a very few bacteria and are stored at ultralow temperatures that do not allow for the growth of microorganisms, hence an ideal assay should detect a very small number of microbes. Apparently, less numbers of *C. perfringens* have secreted little quantity of alpha toxin, insufficient for bringing the colorimetric change.

Our experiments show that even at the very low concentration of alpha-toxin (lecithinase), such as 0.002 (U/l), the developed strip shows a slight colour change, however, a better result was obtained when *C. perfringens* numbers exceeded 100. The assay procedure presented in Table 2 was repeated 20 times in different laboratory

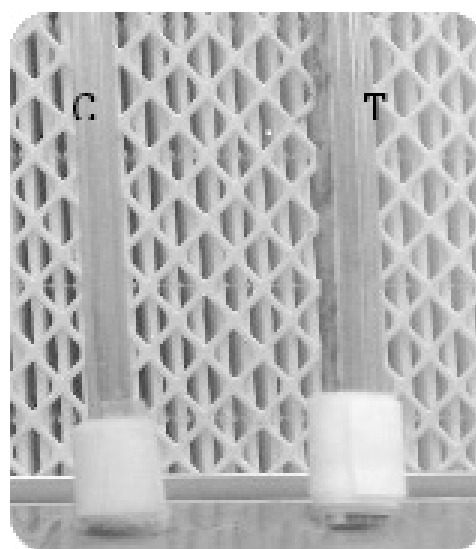


Figure 2. Control flask (C) contained FTB broth inoculated with an *E. coli* culture (100 CFU/ml). Nonappearance of colour reaction in control strips indicates the unambiguous reaction of PNPC with lecithinase and its specificity towards *C. perfringens*.

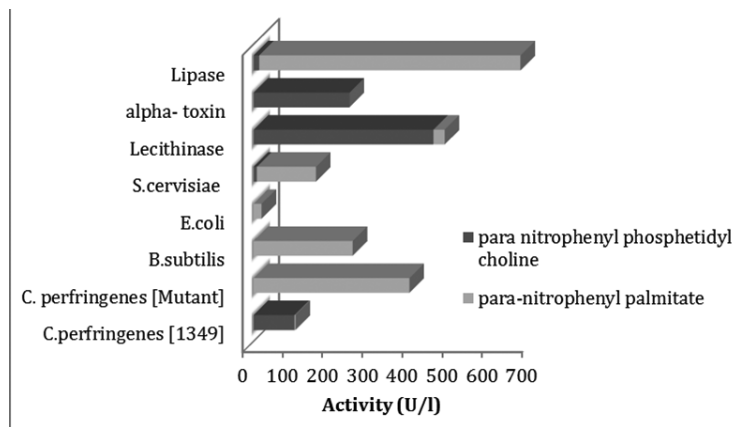


Figure 3. Estimation of lecithinase and lipase activity in different microbial species

and field environments to ensure the accuracy of the assay. The mean values of alpha toxins (lecithinase) are presented in Table 2 as well.

This assay is unequivocally sensitive for targeted pathogenic microbes. It is extremely fast when compared to other, previously reported assays for *C. perfringens*. These assays are either PCR based (which takes at least 4-h for preparation and detection) or are immunoassays which takes longer incubation time (Kanakaraj *et al.*, 1998; Kokai-kun *et al.*, 1994; Amar *et al.*, 2002). In reported assay, the colour development was observed within 30-minutes of incubation when the concentration of alpha-toxin was high. However, in the presence of low concentration of the alpha-toxin, it takes longer for colour development to take place (1-h), thus the optimized time for this assay is 1-h. In addition, the temperature does not exert any significant effect on the colour development reaction, i.e. an increase or decrease by 10°C seems to be insignificant (not shown).

Any procedure is meaningful when it is specific for intended use only. The strip is impregnated with *p*-nitrophenyl phosphatidylcholine, which is a substrate for phospholipase C. PNPC is a natural phospholipid that may be utilized as a substrate by other nonspecific lipases. To evaluate the specificity of this designed substrate only for phospholipase C, the biochemical reaction was evaluated with different microbial species that produced other classes of lipases. Purified lipase, lecithinase (plant source) and alpha toxin (lecithinase from *C. perfringens*) were also used as a control. The results presented in Fig. 3 confirm the specificity of the colour reaction for the designed assay. No significant lipase activity was observed in the reaction vials containing *C. perfringens* in 1% PNPC broth and for *C. perfringens* in para-nitrophenyl palmitate broth. In contrast, the lecithinase mutant strain of *C. perfringens* has shown a significant lipase activity in 1% para-nitrophenyl palmitate broth (PNPB) but no activity in the PNPC broth. Lipolytic enzymes of *C. perfringens* have shown high substrate specificity towards their respective substrates. These results narrow down the chance of false detection, as no activity was detected for any other microbial genus studied in the present study. There are reports in the literature that the secretion of extracellular phospholipase C occurs in many other microbial species. Such reported species are *Pseudomonas* (Sacherer *et al.*, 1994), *Acinetobacter* (Vallenet *et al.*, 2008), and *Bacillus anthracis* (Read *et al.*, 2003), and they can utilize PNPC through phospholipase C secretion. These species might constitute a threat towards the

specificity of this assay for *C. perfringens*, but the complete absence of oxygen in canned food products prevents the growth of the mentioned aerobes. Thus, the canned food product preparation procedure in fact increases the accuracy of the designed assay towards the *C. perfringens* detection. In our analysis, this assay procedure has shown 100% accuracy in developing colour reaction with *C. perfringens* only. However, improper storage of food can allow the growth of other aerobes that might interfere with the detection through production of various metabolites. Hence, the chances of false positive or false negative results cannot be completely eliminated. The result of this assay should be confirmed with another microbiological medium based assay.

#### Effect of storage temperature and microbial load on the enzyme activity; a two-component analysis

Canned food products are generally stored at very low temperature, and this adversity may affect the extracellular lecithinase and make it difficult to detect by the current assay. To check this hypothesis, different numbers of *C. perfringens* were incubated for 24-h at different temperatures in tightly closed screw cap vials kept in the anaerobic chamber. The results show that with increasing storage temperature, the enzyme activity increases up to 100 folds (Fig. 4).

Though temperature significantly affects the lecithinase stability, a higher number of *C. perfringens* cells gave a positive reaction; a detectable enzyme activity was recorded even nearing the freezing temperature of 2°C. The colorimetric detection method is very sensitive as it gives positive colour reaction at very low concentration of microbes, i.e. as little as 100 bacteria. In principle, this assay gives a positive colour reaction by the enzyme-mediated catalysis of PNPC, hence it is extremely important to determine the critical concentration of the enzyme for reaction to occur. This reaction can be obtained at very low concentration of enzyme, 0.002 U/l activity of lecithinase is sufficient for the colorimetric detection. At this concentration the colour is very faint yellow, and a minimum 1000 CFU of *C. perfringens* are considered desirable for detectable colour development. This experiment also demonstrates the significant effect of the stor-

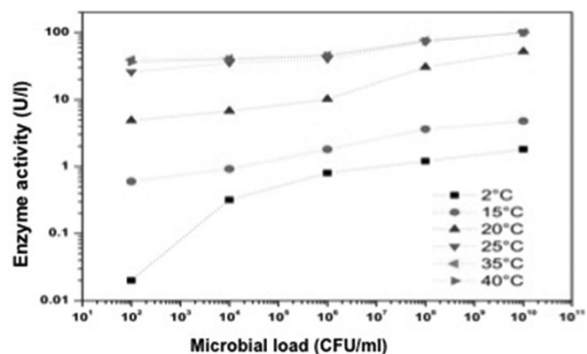


Figure 4. Effect of the storage temperature and the number of *C. perfringens* on the enzyme activity

**Table 3. Study of the efficiency of colour reaction in the presence of different substrate and Clostridia species**

	<i>C. perfringens</i>	<i>C. botulinum</i>	<i>C. tetani</i>	<i>C. difficile</i>	<i>C. sordellii</i>
2% starch	Yes	No	No	No	No
2% peptone	Yes	No	No	No	No
2% egg yolk	Yes	Variable*	No	No	No
2% olive oil	Yes	No	No	No	No
Nutrient broth	Yes	No	No	No	No
FTB	Yes	Variable*	Variable**	No	No
Mineral Medium	No	No	No	No	No

\*The positive reaction was observed for the culture growing below 20°C. \*\*This culture gave positive reaction only in the presence of thioglycollate

age temperature and microbial load that are critical factors for detection.

#### Effect of heterogeneous substrate on color reaction

The genus Clostridia is extremely diverse in nature. It is closely related to many species, which are phylogenetically intermixed with other spore forming and non-spore forming genera that include more than 35 different species (Collins *et al.*, 1994). Most likely, the other clostridium species can give a colour reaction with PNPC too; to investigate the risks of this cross-species reaction, the following experiment was performed. Five different Clostridia species were inoculated in media containing different substrates. A variety of canned food products is available on the market and contains a variety of macromolecules. *C. perfringens* can utilize other substrates by producing another set of enzymes. To evaluate the substrate-dependency of alpha-toxin production, an assay was performed with a variety of substrates. The alpha-toxin production was evaluated in the presence of starch, protein and lipid as a sole substrate and the results of this independent evaluation are summarized in Table 3.

Five Clostridia species were incubated with various substrates at 4 different temperatures i.e. 2°C, 20°C, 37°C, 40°C. This table summarizes the results for the whole temperature range, as no effect of temperature was observed for all cases tested, except three instances noted in the Table 3

The results presented in Table 3 further corroborate the specificity of our color reaction assay. It also sheds light on the pattern of alpha toxin production in *C. perfringens*. *C. perfringens* constitutively produce alpha toxin independent of the nature of substrate and temperature. Our results demonstrate for the first time the temperature dependent alpha toxin production in *C. botulinum*, however, this result does not pose any major threat towards the specificity of our assay as most of the canned food products are stored below 20°C. The results obtained in the FTB medium may pose a constraint toward the species specific detection through this assay as the selected strain of *C. botulinum* and *C. tetani* both gave positive color reaction when grown in FTB. In order to study this constraint in detail, the knockout FTB media were prepared for each individual component. Striking-

ly, in the absence of thioglycollate both of the species gave a negative color reaction. This primary study is not sufficient to establish any relationship between the presence of thioglycollate and alpha-toxin production, however, this study leaves an open-ended question for further research. The review of Hatheway (1990) suggests some interesting factors that are common for more than one species or group. Such common metabolites that increase the pathogenicity of microorganisms are neurotoxins, lethal toxins, lecithinases, oxygen-labile hemolysins, binary toxins, and ADP-ribosyltransferases. This review suggests that the Phospholipase C (lecithinase) production is restricted to only the 2–3 species. The Hatheway and other researchers' results indirectly support the specificity of the assay that we have developed.

#### Effect of microbial consortia on the color reaction

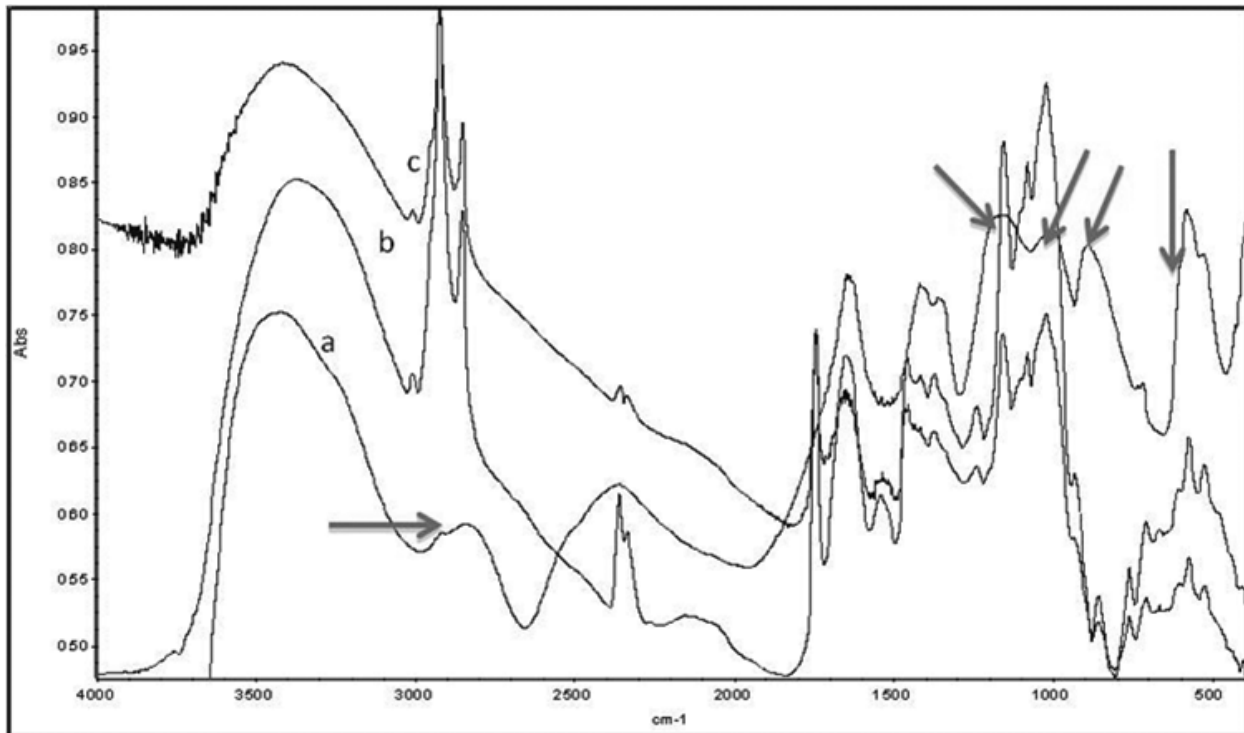
The presence of other bacteria might affect the color reaction. To evaluate the influence of other bacterial species on our assay, a combinatorial approach was used. Two different cultures were incubated in nutrient glucose broth for 72-h at 37°C and were tested in through the design our colorimetric assay. The results are summarized in Table 4.

This study represents the different combinations of bacterial species, mixture of aerobes and anaerobes, mixture of aerobes and facultative anaerobes, mixture of anaerobes and facultative aerobes.

Interestingly, the combination of anaerobes and yeast results in the growth of *C. perfringens* even under anaerobic conditions, which may be the result of

**Table 4. Influence of other microbes and growth conditions on the colour reaction assay**

Condition	Complex	Reaction
Aerobic	<i>C. perfringens</i> + <i>S. cerevisiae</i>	Yes
	<i>C. perfringens</i> + <i>E. coli</i>	No
	<i>C. perfringens</i> + Clostridium species	Yes
Anaerobic	<i>C. perfringens</i> + <i>S. cerevisiae</i>	Yes
	<i>C. perfringens</i> + <i>E. coli</i>	Yes
	<i>C. perfringens</i> + Clostridium species	Yes



**Figure 5. FT-IR spectra of:**

(a) PNPC+lecithinase. (b) PNPC (c) PNPC+lipase single arrow indicates the peak of para-nitrophenyl which is absent in PNPC+lecithinase

the fermentation byproduct (carbon dioxide) produced by the *S. cerevisiae* yeast. It suggests that carbon dioxide produced by yeast results in creating the anaerobic environment for the growth of Clostridia. This result further confirms that this color reaction is not influenced by the presence of other microbes. A combination comprising the *E. coli* and *C. perfringens* has given a negative result in the aerobic environment as predicted, because *C. perfringens* fails to grow in the presence of atmospheric oxygen.

#### FT-IR analysis for validation of the proposed mechanism

The fact that phospholipase C can hydrolyze phosphatidylcholine was reported by many research groups (Sheikhnejad & Srivastava, 1986; Itabe *et al.*, 1994) and the fact that para-nitrophenyl butyrate can serve as a substrate for lipase is broadly reported by many researchers. Itabe *et al.* (1994) has reported the interaction of PNPC with lecithinase and that has greatly inspired the work presented here. The action of lecithinase liberates the para-nitrophenyl from PNPC and this was reported even earlier in the 20<sup>th</sup> century (Thorpe & Miller, 1981). These findings were neglected for a very long period of time and the reported colour reaction was never utilized for the development of a colorimetric assay. To re-confirm these two-decade-old findings, FT-IR analysis was performed (Fig. 5).

Rodrigues and coworkers (2007) has reported FT-IR spectrum of para-nitrophenyl phosphatidyl choline and phosphatidylcholine. The para-nitrophenyl group gave specific peak at the wave number 3000  $\text{cm}^{-1}$  that was found to be completely missing in the sample which was incubated with PNPC lecithinase. The similar peak of para-nitrophenyl was seen in PNPC+ lipase that indicates that the lipase cannot liberate para-nitrophenyl

group from phosphatidylcholine. Pure lecithinase can remove the para-nitrophenyl group from phosphatidylcholine. The FT-IR spectrum suggests the lipase is inducing some change in the functional group of PNPC (multiple blue arrows) but it does not catalyze the hydrolysis of para-nitrophenyl. It further commemorates the specificity of the developed assay.

In summary, our colorimetric assay is reliable and species-specific when performed properly. This assay is designed and optimized for canned food products. The temperature, presence of other bacteria and the nature of food products do not hinder the development of the colour reaction.

#### CONCLUSION

This assay can be employed for all: the solid, liquid and semi-solid food products with little modification in the assay procedure. This study concludes with two open ends for research; one is the effect of thioglycolate on production of the alpha toxin and the second is the effect of temperature on the alpha toxin production by *C. botulinum*. This simple assay can be employed anywhere and by anyone, and the only requirement for development of the colour reaction is the ability to maintain a proper temperature. In a country like India, the atmospheric temperature is always above 30°C, except for the winter and during the night. As mentioned above, at temperatures over 30°C, the production of alpha toxin by *C. botulinum* may occur – this problem needs further investigation. The assay we have developed gave colour reaction at room temperature in India. The research we presented here may open the door for the routine screening of canned food products for *C. perfringens*.

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