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RESEARCH ARTICLE

CONTROLLING TOXIN PRODUCTION (LECITHINASE) BY THE FOOD BORNE PATHOGEN *BACILLUS CEREUS* ISOLATED FROM SOME FRIDGE-KEPTVEGETABLES

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ABSTRACT

In the present study,*Bacillus cereus* previously isolated from local 7daysfridge – kept green pepper and lettuce showed the ability to produce thehighest visible growth, in corn millet broth, after 24hrs incubation at 37 ºC. Thereafter, the growth decreased gradually by increasing the incubation period up to 120 hrs. However, the reverse occurred with the amount of secreted lecithinase enzyme (toxin) where secretion started after 24hrs incubation and then significantly increased gradually to reach the highest amount after 96 hrs incubation. No trend could be observed between incubation temperature and both bacterial growth and toxin production, however the temperature 37 ºC was optimum for both whereas 28 ºC was the minimum. The results revealed the ability of the isolate to grow at a wide pH range extending from 2.6 to 10.6. Adjusting the pH of the corn millet growth medium at acidity (2.6-5.0) or neutrality was followed by low viable colony count, however at alkalinity (8.0-10.5) the count was high. Concerning lecithinase production, the acidic (2.6 – 5.0) or neutral pHs (7.0) was not suitable for toxin production where it stopped completely but the isolate began to produce toxin at alkaline pHs starting from pH 8.6 to 10.6. A little amount of toxin was produced by *B. cereus* in presence of lemon juice in the growth medium while it was completely ceased in presence of acetic acid,a phenomenon which can help in controlling toxin secretion by such contaminating bacteria.

INTRODUCTION

Toxins in food is a widespread hazardous caused by many agents. One of these toxins is microbial toxins which are produced by pathogenic microorganisms such as pathogenic bacteria resulting in health risks (Madigan *et al*., 1997). Actually, only few of these toxins can influence the pathogenicity of the producing organisms (Williams and Clarke, 1998). However, with some food-borne pathogens like *Staphylococcus aureus* and *Bacillus cereus* toxins are performed in the food and the presence of viable bacteria is not required in the disease condition (Varnam and Evans, 1991). *Bacillus cereus* is commonly considered the most troublesome member of the genus *Bacillus* to the food industry. It is spore former food-borne pathogens, produces toxins in the food andsome strains can grow at refrigeration temperatures and spores can withstand high temperatures (Schraft and Griffiths, 1995). Detection of *B. cereus* toxicity in food can be achieved through lecithinase enzyme activity (Phospholipase C) which hydrolyses lecithin on egg yolk agar plates (Oladipo *et al*, 2008; Samanta, 2012). Lecithinase is a potent toxin and causes lysis of red blood cells, myocytes, fibroblasts, platelets, and leukocytes (Shetty *et al*., 2009) in addition to disruption of cell membrane (Todar, 2002). Actually, lecithinase activity is a special tool for detection of *B. cereus* (Cecilie *et al*., 2005), *B. licheniformis, B. brevis* (Kashid and Ghosh, 2010) and other Bacillus species (Guinebretie` re *et al*., 2013). Food contaminated with *Bacillus cereus* and *B. licheniformis* would contain lecithinase within a short period of 1 and 2 h, respectively (Kashid and Ghosh, 2010).The goal of the present study was directed to evaluate the effect of some factors on lecithinase (toxin) production by *Bacillus cereus* in a hope to reduce its release from cells.

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MATERIALSAND METHODS

Organism

Bacillus cereus previously isolated from 7 days- fridge kept green pepper and lettuce, identified by biochemical tests and confirmed with phoenix system and showed the highest production for lecithinase on egg-yolk agar plates (R. Abuseif, Taibah University, AL-Madinah AL- Munawarah, K.S.A, MSC thesis) was used in this study.

Effect of different factors on lecithinase production and viable colony count of *B.cereus*

Effect of incubation period

a. Lecithinase enzyme (LE) activity

The test was carried out according to Oladipo *et al*. (2008) with slight modification where the broth contained 10% corn millet

replacing Ogi in the original broth. After equal distribution in test tubes (10 ml / tube) and sterilization, the broth was inoculated with 1ml of *Bacillus cereus* culture (24 hr. age) at the proper dilution, incubated at 37 °Cfor different time intervals (24, 48, 72, 96 and 120 hrs) (Nandy *et al.,* 2009) then centrifuged at 2500 rpm for 15 min (MPW-350R High Speed Brushless Centrifuge) to obtain cell free filtrate (CFF). One hundred µl of the CFF was introduced into wells (10 mm diameter) made centrally in egg-yolk agar (EYA) plates (Kashid and Ghosh, 2010) and incubated for 24 hr. at 37 °C. The developed opaque zones were measured (mm), means were calculated and taken as a criterion for lecithinase (LE) activity (toxin production).

b. Viable colony count

One ml of the previous culture broth before centrifugation was aseptically diluted and streaked on nutrient agar plates, and incubated at 37 °C for 24 hr. After incubation, the number of viable colonies was counted (Oladipo *et al*., 2008), means were calculated and the $\log_{10}$ of the CFU/ml of viable count was estimated.

Effect of temperature

Tubes containing 10 ml of corn millet broth were inoculated separately with 1 ml of *B. cereus*culture (24 hr. age) at the proper dilution and incubated at 6, 28, 37 and 45 °C for 5 days. Both of toxin production (lecithinase activity) and viable colony count were detected, as previously mentioned.

Effect of pH

Citrate-Phosphate Buffer at the pH range from 2.6 to 7.0 (Oladipo*et al*.,2008) and Glycine-Sodium Hydroxide buffer at the pH range 8.6 to 10.6 (Vogel and Bierman, 1967)were used for adjusting the pH of the corn millet medium at 2.6, 5.0 , 7.0, 8.6, 9.0, 10.0 and 10.6. The medium was dispensed in test tubes (10ml, each) then inoculated with the investigated isolate and after incubation at 37 °C for 5 days toxin production and viable colony count were detected.

Effect of lemon juice and acetic acid on toxin production

One ml of either purelemon juice or acetic acid (6%) was aseptically added to sterile corn millet broth (l0 ml), the pH of the mixtures were 4.34 and 4.64, respectively. The tubes were inoculated with *B. cereus,* incubated at 37ºC for 5 days then toxin production through LE activity was detected.

Statistical analysis

All the treatments were performed in triplicates and Statistical analysis was evaluated using SPSS software programmed one-way ANOVA to obtain the least significant difference (L.S.D) at 5%.

RESULTS

Factors affecting growth and production of lecithinase enzyme

Incubation period

The effect of incubation period on growth response of *B. cereus* and toxin production was monitored (Table 1). The isolate showed the ability to produce the highest prominent visible growth after 24 hr. However, the gradual prolongation of incubation period up to 48, 72 and 96 hr. was followed by aconcomitant gradual inhibition in the growth. Meanwhile, a faint growth was observed after 120 hr. ($\log_{10}$ 5.72) but the number of viable colonies was decreased compared to the initial size of inoculum (0 time, $\log_{10}$ 5.89). Concerning lecithinase production by *B. cereus,* the enzyme secretion started after 24 hr. incubation where the diameter of the opaque zone was 27 mm and significantly ($P \leq 0.05$) increasedgraduallyby increasing the incubation period up to 96 hr. where the diameter of the obtained opaque zone reached 40 mm. However, increasing the incubation period up to 120 hr. was followed by a reduction in enzyme production (diameter of opaque zone36 mm) but still more than the lower incubation periods from 24 to72 hr.

Table 1. Effect of incubation period on growth and lecithinase production by *B. cereus* after incubation for 5 days at 37C°

Incubation period	Bacterial growth (Log_{10})	Lecithinaseproduction (mm)
0	5.89	0.0
24	6.36	27
48	6.27	30
72	6.18	32
96	6.16	40
120	5.72	36
LSD at 5%		**0.100**

Incubation Temperature

Table (2) showed that there was no general trend could be observed between incubation temperature and both bacterial growth and toxin production. When *B. cereus* incubated at 6 ºC for 5 days the recorded growth was 5.58 ($\log_{10}$ CFU/ml) and after incubation at 28 ºC the growth decreasedto 5.08. However, increasing incubation temperature to 37 ºC was followed by an increase in the visible growth (5.92) but at 45 ºC colony count was reduced to reach 5.39. Similar trend was followedwith LE production by *B. cereus* and it was capable to produce the enzyme at all growth temperatures. The significant LE production was observed at 37 ºC (33 mm) while the least was recorded at 28 ºC (17 mm).

Table 2. Effect of incubation temperature on growth and lecithinase production by *B. cereus* after growth for 5 days at 37C°

Temperature C°	Bacterial growth ($\log_{10}$)	Lecithinase production (mm)
6	5.58	20
28	5.08	17
37	5.92	33
45	5.39	19
LSD at 5 %		**0.010**

Medium pH

The result of the effect of medium pH on growth of *B. cereus* revealed the ability of the isolate to grow at a wide pH range extending from 2.6 to 10.6 (Table 3). Actually, viable colony count was low at acidic pHs (2.6 – 5) and high at either neutral or alkaline pHs (8.0-10.6). The highest recorded growth (6.26) was observed at pH 10.0. Concerning LE production, the acidic (2.6 -7) or neutral (7) pHs was not suitable for toxin production where it stopped completely. However, the isolate began to

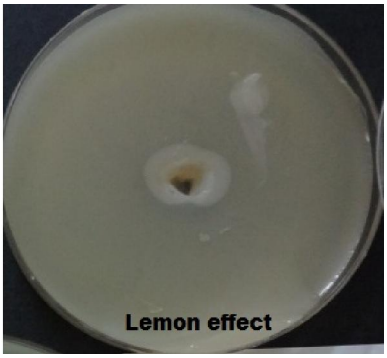
produce toxin at alkaline pHs starting from pH 8.6, and increased gradually (P≤0.05) at pH 9 and pH 10 where the diameter of opaque zones were 25, 29 and 32 mm, respectively. Increasing the pH up to 10.6 was followed by a slight reduction in enzyme output (31 mm diameter of opaque zone).

Table 3. Effect of medium pH on growth and lecithinaseproduction by *B. cereus* after 5days incubation at 37C°

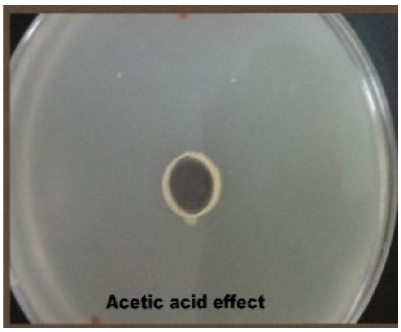
pH	Bacterial growth (log_{10})	Lecithinaseproduction (mm)
2.6	5.76	0
3.0	5.83	0
5.0	5.85	0
7.0	6.14	0
8.6	6.06	25
9.0	6.03	29
10.0	6.26	32
10.6	5.92	31
LSD at 5%		0.211

Effect of lemon juice and acetic acid on toxin production

The result of the effect of pH on toxin production by *B. cereus* was urged to investigate the effect of lemon juice and acetic acid on the production. Figure (1) showed that a small amount of toxin (lecithinase enzyme) was produced by *B. cereus* in presence of lemon juice (9 mm) while it was completely ceased in presence of acetic acid.



Lemon juice



Acetic acid

Fig (1). Effect of lemon juice and acetic acid on lecithinase production by *Bacillus cereus*.

DISCUSSION

Over the years, food is considered to be the main source for food-borne diseases in different countries due to contamination by pathogenic organisms (Boonestroo *et al*., 1993). The growth of these spoilage and toxigenic organism is, therefore, a strong danger in foods that depend on mild heat treatment and refrigerated storage to keep (Kenneth, 2008; Kashid and Ghosh, 2010). In the current study, the highest viable colony count of *Bacillus cereus* was observed after 24 hr. incubation then the count decreased with growth prolongation. This trend was reversed with LE secretion where it started after 24 hr. growth and increased gradually by increasing the incubation period up to 96 hr. Likely, *Klebsiellaaerogenes* and *Enterobacteraerogenes* produced detectable toxin after 24 hr. incubation (Oladipo *et al*., 2008). This could be explained by the fact that the amount of lecithinase produced was small in young cultures during proliferation (Williams and Clarke, 1998) and increased to a maximum at the stationary phase when active cell division stopped. However, increasing the current incubation period up to 120 hr. was followed by a reduction in both growth and enzyme yield of *B. cereus* which accompanied cell lysis (AL-Qadiri *et al*., 2008).

The results revealed that *B. cereus* exhibited a visible growth and toxin production at all tested incubation temperatures even at refrigeration (6 ºC) with optimum at 37 ºC and minimum at 28 ºC. Similar optimum temperature was obtained by Coffey *et al.* (1996) and Oladipo *et al*. (2008). In this regard many Bacillus isolates, including *B. cereus*, showed ability to grow and to produce lecithinase at refrigeration temperatures in the range of 10-12C°, or even 4-6C° (Carlin *et al*., 2006; Coorevits *et al*., 2010) and at maximum temperature 50C° or 55C° (Trojanowska *et al*., 1996). Growth at high temperatures might be due to the heat resistance of the spore and the ability of the vegetative cells to produce extracellular enzymes (Bellow *et al*., 2007; Floristean *et al*., 2007).

It is worthy to mention that *B. cereus* could grow at a wide pH range extending from 2.6 to 10.6 but growth at alkaline pHs was more luxurious than at acidic ones. In this connection, Goepfert and Kim (1972) reported that *B. cereus* showed growth in media with pH range lies between 4.9 to 9.3. Interestingly, toxin production was stopped completely when the medium was neutral or acidified and production was traced only in alkaline pHs. Similarly, Nakamura *et al*. (1968) reported that lecithinase was readily inactivated by acid pH, but was more resistant to alkaline ones. Moreover, Coffey *et al*. (1996) reported that pH values lower than 6.5 were observed to reduce slightly bacterial growth but significantly inhibited lecithinase production. Oladipo *et al*. (2008) reported that pH 3 and 5 were less suitable for toxin (lecithinase) elaboration by the tested bacteria, while they were able to grow at different pH ranges. Fortunately, treatment of corn millet broth with either acetic acid or lemon juice negatively affected toxin (lecithinase) production by *B. cereus* where it was completely arrested by the former and greatly reduced by the latter; an observation which can be exploited in protecting food from bacterial contamination and toxin production.

Conclusion

Finally, it could be concluded that long keeping of vegetables in the fridge is not preferred because it becomes exposed to contamination by toxic bacteria. However, the use of acetic acid or lemon juice in processed food can help in reduction of

toxin secretion by contaminating bacteria leading to minimizing disease risk.

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