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Full Length Research Paper

Influence of concentrated supernatants from *Bacillus cereus* var. *Toyoi* strain on the metabolic activity of vero cells

Abdelhak Darbouche*
Institute of Biology, Department of Microbiology, University Khenchela; Route de El Hamma Khenchela, (Algeria).

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This report summaries results concerning an influence of concentrated bacterial supernatant from *Bacillus cereus* var. *Toyoi* CNCM I-1012/NCIMB 40112 (probiotic-Toyocerin) on Vero cells. To test the influence of the supernatant on the metabolic activity of Vero cells, supernatant was concentrated 5-fold using the "Centriprep Kit concentrator" concentration system with a cut off of 3.000 Da. and were incubated for 2 h on Vero cell monolayers. The bacterial supernatant was tested in triplicate in a dilution of 1:8 (12.5 ml supernatant + 87.5 ml RPMI) on Vero cells (Vero I ATCC CCL 81). As negative controls, wells received 5-fold concentrated supernatant from *Bacillus thuringiensis* T 26001 and BHI + 1% Glucose. *Bacillus cereus* 1230 was used as positive control. The negative metabolic effects/cytotoxic effects was objectified through the use of MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromid]. This assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells (Cory et al., 1991; Mosmann, 1983). The solublized formazan product was spectrophotometrically quantified using an ELISA reader.
The assays revealed that concentrated supernatants of the probiotic strain *B. Cereus* var. *Toyoi* reduced the metabolic activity of Vero cells.

Key words: *Bacillus cereus* var. *Toyoi,* Vero cells, bacterial supernatant, metabolic activity

INTRODUCTION

Bacillus cereus var. *toyoi* is not a member of the normal animals gut flora, but is a common soil inhabitant. A number of studies have assigned probiotic characteristics to this particular Bacillus strain (*Bacillus cereus* var. *Toyoi* CNCM I-1012/NCIMB 40112; ToyoCerin1), and it has been authorized in the EU for use as a probiotic feed additive for sows and piglets and several other farm animal species (SCAN, 2000). In animal studies with various strains of *B. cereus*, positive effects such as increased weight gain, improved feed conversion ratios and lower mortality rates of piglets have been reported (Kirchgessner et al., 1993; Alexopoulos et al., 2001), and *B. cereus* var. *toyoi* was correlated with a reduced incidence of post-weaning diarrhea (Taras et al., 2005; Scharek et al., 2007).

*Corresponding author adarbouche@hotmail.com

In this communication we assessed the effects of concentrated bacterial supernatant from *Bacillus cereus* var. *Toyoi* CNCM I-1012/NCIMB 40112 (probiotic-Toyocerin) on Vero cells.

MATERIALS AND METHODS

Bacterial strains tested

The cytotoxicity assay was carried out with the following bacterial strains:

1- *Bacillus cereus* 1230 (**positive** enterotoxic strain provided by P.E. Granum Oslo)
2- *Bacillus cereus* var. *toyoi* CNCM I-1012/NCIMB 40112 (probiotic-Toyocerin)
3- *Bacillus thuringiensis* T 26001 (**negative** enterotoxic strain provided by P.E. Granum Oslo)

Cell lines, media and buffer solutions

BHIG: Brain Heart Infusion + 1% glucose (Oxoid, Basingstoke, England)
Vero cells I (vero I): ATCC CRL 1587 (provided from the Institut für Hygiene und Infektionskrankheiten der Tiere, Giessen, Germany)
Cell culture medium: see A4.
Stop solution: 10% SDS in ddH_2O (Sigma Chemical co., St. Louis, U.S.A.)
Phosphate buffer: Formula (g/l)
(10 x PBS pH 7.4) NaCl 80.0 (Carl Roth GmbH + co, Karlsruhe, Germany) KCl 2.0 (FERAK, Berlin, Germany) Na_2HPO_4 x $2H_2O$ 7.6 (E Merck, Darmstadt, Germany) KH_2PO_4 2.0 (E Merck, Darmstadt, Germany)
MTT: 5mg/ml in 1x PBS (Sigma Chemical co., St. Louis, U.S.A.) (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromid)

Growth conditions and toxin production

The growth conditions of all strains were chosen like described in the SCAN report (5). A single colony of each bacterium (*Bacillus*) was inoculated into 50 ml of Brain Heart Infusion broth with 1% glucose (BHIG) and then cultured overnight at 32°C with shaking (100 rpm). After overnight culture, 1 ml of each cultivated broth was transferred into 50 ml of BHIG and incubated at 32°C for 6 h with shaking (100 rpm).
The OD_{600nm} was measured using the spectrophotometer "Ultrospec 3000" (Amersham Pharmacia Biotech, Freiburg. Germany), the cells were separated by centrifugation (5000g at 4°C for 20 min) and the supernatant was filtered through 0.22 µm pore-size filters (Millipore co, U.S.A). Proteins of the culture supernatant were concentrated 5-fold using the Centriprep Kit YM-3 MWCO: 3.000 (Millipore Co, U.S.A). Immediately after concentration of the supernatants they were used for the Vero cell cytotoxicity test.

Cell lines and preparation of medium

Vero cells (African green monkey kidney; Vero I, see A2) were cultivated in tissue culture flasks with RPMI 1640 medium (Gibco, NY, U.S.A), supplemented with 10% foetal calf serum (Gibco, NY, U.S.A), 0.75 mM L-glutamine (Gibco, NY, U.S.A.), 40 µg/ml penicillin/streptomycin (Gibco, NY, U.S.A), and 1 µg/ml amphotericin B (Gibco, NY, U.S.A).

Cytotoxicity assay

The cytotoxicity assay was performed as described by Konowalchuk et al. (3) and Dalrymple and Gentry (1). Confluent monolayers were removed with trypsin-ED'I'A (Gibco, NY, U.S.A), resuspended to approximately 4 x 10^5 cells/ml in RPMI and 0.l-ml samples were pipetted into each well of a 96-well microtiter plate. After incubation at 37°C in 5% CO_2 for 72 h, the medium was replaced with 1:8 (12.5 µl supernatant + 87.5 µl RPMI; 100 µl end volume) of the

concentrated bacterial supernatants in RPMI and was added to each well. As negative controls, some wells received only RPMI or PBS. 1% SDS (in PBS) was used as positive control. Each sample was tested in triplicate. Cytotoxic effects on Vero cells were detected by monitoring the metabolic activity of the cells through the use of MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromid).

Before the MTT test started the media containing the bacterial culture filtrates were removed and each well was washed for 2-3 times with 0.89% NaCl. The medium was replaced with 150 µl 1 x PBS + 25 µl MTT (5mg/ml in PBS). Then the Vero cells were incubated with the yellow MTT solution for 4 h (e.g. 37°C, 5.0% CO_2, shacked). After this incubation period, purple formazan salt crystals are formed. These salt crystals are insoluble in aqueous solution, but will be solubilized by adding 100 µl SDS stop-solutions (10% SDS in ddH_2O) and incubating the plates overnight in a humidified atmosphere (e.g. 37°C, 5.0% CO_2, shacked). The solubilized formazan product was spectrophotometrically quantified using an ELISA reader (Dynatech MR5000; Dynex Technologies GmbH).

An increase in number or activity of living cells results in an increase in the total metabolic activity in the sample. The wavelength to measure the optical density (OD) of the Formazan product was 550 nm. Thus the higher the OD, the higher is the metabolic activity of the respective Vero cells.

A negative metabolic effect/cytotoxic effect of supernatants from the 3 test strains was calculated as recommended by the report from the SCAN (2000).

After substraction of the value for background $OD_{550\text{-}690nm}$ the metabolic activity was calculated: ($OD_{550\text{-}690nm}$ for Vero cells without "toxin" - $OD_{550\text{-}690nm}$ test sample) x 100/$OD_{550\text{-}690nm}$ for Vero cells without "toxin" added.

If the $OD_{550\text{-}690nm}$ of the test sample was less than 80% of that of the Vero cells without "toxin", the test sample was considered as "toxin" positive.

RESULTS

Growth of the bacterial strains:

The following tables list the optical densities of the bacterial cultures, immediately before they were used for preparation of the respective supernatants.

Strain	OD_{600nm}
1. *B. cereus* 1230	2.066
2. *B. cereus* var toyoi	1.968
3. *B. thuringiensis* T26001	2.005

Table 1. Results of the OD measurements (600 nm) of the different *Bacillus* strains grown **6 h** at 32°C and 100 rpm in BHI + 1% glucose (BHIG) (as suggested in the "SCAN" report)

Results of the cytotoxicity assays

Vero cells were incubated with supernatants from the 3 strains for **2 h**. As 12.5 µl of a 1:5 concentrated supernatant was tested, the final concentration is nearly identical to the one recommended by the SCAN (2000)**.** A metabolic activity of Vero cells incubated with supernatants from the test strains less than 80% of the negative control cells (Vero cells incubated with RPMI or PBS, respectively) was regarded as yielding cytotoxic effects like described in the SCAN report (2000).

Table 2. Optical Density ($OD_{550\text{-}690nm}$) measured (in triplicate) after cytotoxicity test on Vero cells I with the appropriate supernatants dilutions of 3 strains of Bacillus (*B. cereus* 1230, *B. thuringiensis* and *B. cereus* toyoi)

	$OD_{550\text{-}690nm}$ (Average) 1:8 (l2.5µl+87.5µl)
B. cereus **1230 (+)**	**0.005**
B. thuringiensis **(-)**	0.396
BHI-G	0.377
B. cereus toyoi	**0.190**

Control + (1% SDS) $0.004 \pm 1.0 \times 10^{-4}$
Control – (1 x PBS) $0.435 \pm 25 \times 10^{-3}$
80 % metabolic activity = $OD_{550\text{-}690nm}$ 0.348; values < 0.348 are considered to be toxic
Framed OD: metabolic activity less than 80 % (<**$OD_{550\text{-}690nm}$** 0.348) of the negative control cells
(+) positive control strain (*B. cereus* 1230); (-) negative control strain (*B. thuringiensis* T 26001) and BHI-G
Note: All S.D. (standard deviation) ranged from 10^{-3} to 10^{-4}

CONCLUSION

A negative metabolic effect/cytotoxic effect of supernatants from the 3 test strains (*Bacillus cereus* var. *toyoi*, *Bacillus cereus* 1230 and *Bacillus thuringiensis* T 26001) was calculated as recommended by the report from the SCAN (2000).
If the $OD_{550\text{-}690nm}$ of the test sample was less than 80% of that of the Vero cells without "toxin", the test sample was considered as "toxin" positive.
The assays revealed that concentrated supernatants of the probiotic strain *B. cereus* var. *toyoi* reduced the metabolic activity of Vero cells.
The ammonium sulphate precipitation method suggested by SCAN (2000) was established in our laboratory, but unfortunately the protein concentrations varied substantially from batch to batch (data not shown). The ammonium sulphate concentration at which a certain protein will precipitate is dependant from the number and position of the polar groups, the molecular weight of the protein, the pH value of the solution, and the temperature at which the precipitation is carried out (Bell et al., 1983). Furthermore, removal of the remaining ammonium sulphate by dialysis and the consequent renaturation of the proteins to their native conformation form are not always obvious. To overcome this problem we used another concentration system where proteins in general retain their native form. We utilized a concentration system with a cut off of 3.000 Da., causing substances yielding smaller molecular weights to vanish from the toxin preparation. While the loss of smaller substances clearly is a disadvantage, by this method not only proteins are concentrated, but also fatty acids and sugars will be maintained. These substances may also influence the test result, as toxins of non-protein nature have also been described, i.e. the emetic toxin of *Bacillus cereus*. However, the enterotoxins mentioned in the SCAN-report (2000) for Bacillus are larger than 3 kDa in molecular weight, thus they should be detected by this method.

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