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Inhibition of bongkrek acid and toxoflavin production in tempe bongkrek containing *Pseudomonas cocovenenans*

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Tempe bongkrek was prepared from partially defatted coconut by fermentation with the mould *Rhizopus oligosporus*. The addition of 2% sodium chloride decreased but did not inhibit formation of bongkrek acid by *Pseudomonas cocovenenans* strain ITB in either coconut culture medium (CCM) or tempe bongkrek, while 2% sodium chloride or acidification of CCM to pH 4·5 with acetic acid inhibited formation of toxoflavin. Toxin formation by three strains of *Ps. cocovenenans* in tempe bongkrek was inhibited by the combination of 2% sodium chloride and reduction of pH to 4·5 with acetic acid. Mycelial growth of *R. oligosporus* was sometimes affected by toxin production by *Ps. cocovenenans*, but not by the addition of 2% sodium chloride and/or acidification of CCM to pH 4·5 with acetic acid.

Tempe bongkrek, one of many kinds of Indonesian fermented food, is produced by fermenting partially defatted coconut with the mould *Rhizopus oligosporus*. The coconut material sometimes may be mixed with soybean presscake from the manufacture of tofu or soybean curd. Tempe bongkrek sometimes is contaminated by *Pseudomonas cocovenenans* which can produce at least two poisonous substances, bongkrek acid (BA) and toxoflavin (TF). Consumption of toxin-containing tempe bongkrek can be fatal (van Veen 1966; Arbianto 1979). Both toxins are hazardous to human health, with bongkrek acid more toxic than toxoflavin. Since 1951 there have been nearly 1000 fatalities in Central Java, Indonesia from consumption of tempe bongkrek. In a fatal bongkrek food poisoning outbreak in Java in February 1988, about 200 victims were seriously poisoned and at least 14 died (Anon. 1988). Bongkrek acid interferes with glycogen metabolism and mobilizes glycogen from the liver, resulting in hyperglycaemia which is followed by a fatal hypoglycaemia (van Veen 1966). The blood sugar level can be restored by glucose injection; it gives some relief but does not prevent death (Welling *et al.* 1960).

Traditional attempts to inhibit the growth of *Ps. cocovenenans* in tempe bongkrek have been made for many years. Simple materials, such as acid-containing leaves of plant species that grow wild in the area where the bongkrek problem occurs (i.e. Central Java) and many chemicals have been used. When the coconut-containing material before inoculation with the mould was acidified to a pH of about 5·5 by mixing with acid containing leaves of *Oxalis* spp. *Ps. cocovenenans* scarcely grew although the mould did. However, *Oxalis* leaves give the tempe an undesirable dark colour that explain why consumers have not made more widespread use of this simple safety measure, although a dried extract of leaves of *O. sepium* was more successful (Ko & Kelholt 1981). Ko *et al.* (1979) suppressed toxin formation in tempe bongkrek without undesirable colour changes by adding 1·5–2·0% sodium chloride to the raw material before mould fermentation.

Glucose was suitable for growth of the bacterium but not for BA production. Glycerol and

organic acids, particularly oleic acid, were suitable for both growth and BA production. Ko & Kelholt (1981) showed that *R. oligosporus* under certain conditions has a suppressing effect on toxin production by *Ps. cocovenenans* in tempe bongkrek. Ko (1985) reported that the most favourable conditions for optimum growth and production of toxin by *Ps. cocovenenans* are also the conditions under which tempe bongkrek is manufactured at the village level.

The objective of the present study was to examine the effects of both acidification and addition of sodium chloride on toxin production by *P. cocovenenans* in a coconut culture medium and in tempe bongkrek.

Materials and Methods

COCONUT CULTURE MEDIUM

Fresh coconuts were purchased from a supermarket in Sydney, NSW. Coconut culture medium (CCM) was prepared from partially defatted coconut as described by Ko *et al.* (1979).

MOULD CULTURE

A pure culture of *R. oligosporus* strain BT_3K_1 was obtained from Dr Sutardi, Faculty of Agricultural Technology, Gadjah Mada University, Jogjakarta, Java. The culture was maintained on slants of Potato Dextrose Agar (PDA, Oxoid) at 4°C. Before each experiment, the mould culture was transferred to a PDA slant and incubated for 7 d at 30°C.

BACTERIAL CULTURES

Strains of *Ps. cocovenenans* were obtained from the following sources: (1) Ir.I. Sasmitamihardja, Department of Chemical Engineering, Bandung Institute of Technology, Bandung, Java; (strain ITB)—this strain was used in all experiments in this study; (2) the National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, UK; this strain (NCIB: 9450), referred to as strain NCIB, was used for a comparative study; (3) Dr Ko Swan Djien, Landbouwhogeschool De Centrale Dienst, Biotechnion, De Dreijn 12, Wageningen, The Netherlands; this strain (LMD 38 18), referred to as strain LMD, was also used for a comparative study. Cultures were maintained in 1% soy peptone (SP, Oxoid) at 30°C and transferred weekly to a new medium. For each experiment 1·0 ml of stock culture was transferred to 100 ml SP and incubated at 30°C for 24 h; such cultures contained about 10^6 cells/ml. Serial dilutions of this culture in 0·9% sodium chloride solution were used for inoculations. Inoculated levels are shown in the Tables.

CHEMICALS AND SOLVENTS

Bongkrek acid solution in 1 N ammonia (approx. 50 μg/ml) and toxoflavin in aqueous solution (289 μg/ml) were obtained from Dr Ir. M.I.X. Mager, Biochemical and Biophysical Laboratory, Delft University of Technology, Delft, The Netherlands. All solvents and chemicals used were analytical reagent quality and methanol was chromatographic grade.

PREPARATION OF TEMPE BONGKREK

CCM (45 g) was autoclaved for 15 min at 121°C, cooled and inoculated with *R. oligosporus* spores by mixing it with the appropriate dilution of mould culture with a glass rod in a glass jar. The inoculated CCM was poured into plastic Petri dishes (90 × 15 mm) and incubated at 30°C for 48 h.

PREPARATION OF TEMPE BONGKREK CONTAINING *Pseudomonas cocovenenans*

Each portion of 45 g sterilized CCM was inoculated with 1 ml of *Ps. cocovenenans* culture and 4 ml of spore suspension of *R. oligosporus* containing appropriate levels of organisms or spores as determined by plate count. The inoculated CCM (original pH 6·9) was adjusted to either pH 5·5, 5·0 or 4·5 by addition of 10% solution of acetic acid; or 20% sodium chloride solution was added to a final concentration of 0·6, 1·0 or 2·0%. For some experiments a combination of treatments was used. Duplicate portions (20 g) of the inoculated and treated CCM were filled into plastic Petri dishes and incubated at 30°C for 48 h. The contents of each dish were transferred separately to a 300 ml glass jar for toxin extraction and high performance liquid chromatographic (HPLC) analysis. Toxin production by *Ps. cocovenenans*

Table 1. Mean toxin production (mg/g) by *Pseudomonas cocovenenans* strains ITB, NCIB and LMD in coconut culture medium during incubation at 30°C

Strain	Initial number of *P. cocovenenans* (cells/g CCM)	Incubation time (h) 24 BA	24 TF	48 BA	48 TF	72 BA	72 TF	96 BA	96 TF
ITB	$1{\cdot}2 \times 10^6$	0·08*	U	1·45	0·05	1·55	0·05	1·80	0·07
NCIB	$2{\cdot}7 \times 10^6$	0·02	U	1·70	U	1·15	U	1·40	U
LMD	$2{\cdot}9 \times 10^6$	0·90	0·05	2·60	0·02	2·85	0·03	3·15	0·05

CCM, coconut culture medium; BA, bongkrek acid; TF, toxoflavin; U, undetected.
* Means of 3 analyses.

during incubation of CCM at 30°C was examined after 24, 48, 72 and 96 h.

EXTRACTION AND HPLC DETERMINATION OF BONGKREK ACID AND TOXOFLAVIN CONCENTRATIONS

BA was extracted by the method described by Lijmbach (1969) and TF was extracted by the method described by van Damme *et al.* (1960) and determined quantitatively by HPLC (Voragen *et al.* 1982). The ether extract of BA was evaporated to dryness in a stream of nitrogen gas, and the residue redissolved in methanol, filtered through a pressurized Millipore Swinnex 13 ultrafilter (Sartorius membrane Type 11306, pore size 0·45 μm) and BA determined in a 20 μl sample by a Waters high performance liquid chromatograph, equipped with a Radial pak C_{18} column. BA-containing samples were eluted with a methanol:water:acetic acid (70:29:1 v/v/v) mixture at a flowrate of 2 ml/min. The compound was detected (model 480 Lambda-Max detector) by u.v. absorption at 267 nm. The aqueous solution of TF was ultrafiltered (pore size 0·45 μm) and the clear filtrate analysed by HPLC. Toxoflavin was eluted with a methanol:water:acetic acid (8:91:1 v/v/v) mixture with a flowrate of 1·6 ml/min. The compound was detected by u.v. absorption at 258 nm. Concentrations of BA and TF (mg/g dry weight) were determined by reference to results from samples spiked with pure BA or TF. The limits of detection of BA and TF were less than 10 μg/g CCM or tempe bongkrek.

Results

TOXIN PRODUCTION IN CCM CONTAINING *Pseudomonas cocovenenans*

Effect of incubation time

Incubation of CCM for periods up to 96 h produced marked increases in BA content (Table 1)

Table 2. Bongkrek acid and toxoflavin production (mg/g) by *Pseudomonas cocovenenans* strain ITB after 48 h incubation at 30°C in coconut culture medium treated with sodium chloride and acetic acid

pH	Sodium chloride concentration (%)	Initial number of *P. cocovenenans* (cells/g CMM)	Bongkrek acid	Toxoflavin
6·9	0	$1{\cdot}2 \times 10^6$	1·50*	0·05
6·9	0·6	$1{\cdot}2 \times 10^6$	1·40	0·03
6·9	1·0	$1{\cdot}2 \times 10^6$	0·80	0·02
6·9	2·0	$1{\cdot}2 \times 10^6$	0·05	U
5·5	0	$1{\cdot}2 \times 10^6$	0·70	0·05
5·0	0	$1{\cdot}2 \times 10^6$	0·09	0·03
4·5	0	$1{\cdot}2 \times 10^6$	0·05	U
5·5	1·0	$4{\cdot}3 \times 10^6$	0·05	U
5·5	1·0	$1{\cdot}2 \times 10^6$	U	U
5·0	2·0	$6{\cdot}0 \times 10^6$	U	n.d.
4·5	2·0	$6{\cdot}0 \times 10^5$	U	n.d.

* Mean of 3 determinations.
U, undetectable; n.d., not determined.

Table 3. Toxin production (mg/g) by *Pseudomonas cocovenenans* and growth of *Rhizopus oligosporus* in tempe bongkrek containing sodium chloride and acetic acid after 48 h incubation at 30°C

Bacterial strain	pH	NaCl concentration (%)	Initial number of *R. oligosporus* (spores/g)	Initial number of *P. cocovenenans* (cells/g)	Mould growth	Bongkrek acid	Toxoflavin
ITB	6·9	0	—	$1·2 \times 10^6$	n.a.	1·50	0·05
	6·9	0	—	$2·9 \times 10^6$	n.a.	2·40	0·08
	6·9	0	$4·2 \times 10^4$	—	+	U	n.d.
	6·9	0	$4·2 \times 10^4$	2·9	+	0·07	n.d.
	6·9	0	$4·2 \times 10^4$	$2·9 \times 10^4$	+	0·20	n.d.
	6·9	0	$4·2 \times 10^4$	$2·9 \times 10^5$	−	0·60	n.d.
	6·9	0	$4·2 \times 10^4$	$2·9 \times 10^6$	−	1·90	n.d.
	6·9	0	$6·7 \times 10^4$	$1·2 \times 10^6$	−	0·85	0·03
	5·5	0	$6·7 \times 10^4$	$1·2 \times 10^6$	+	0·15	U
	5·0	0	$4·0 \times 10^4$	$1·2 \times 10^5$	+	U	n.d.
	4·5	0	$4·0 \times 10^4$	$1·2 \times 10^5$	+	U	n.d.
	6·9	1·0	$6·7 \times 10^4$	$1·2 \times 10^6$	+	0·20	0·05
	6·9	2·0	$5·0 \times 10^4$	$2·0 \times 10^6$	+	0·04	n.d.
	5·5	1·0	$1·8 \times 10^4$	$4·3 \times 10^6$	+	U	U
	5·5	2·0	$7·5 \times 10^7$	$1·3 \times 10^9$	+	U	U
	4·5	1·0	$7·5 \times 10^7$	$1·3 \times 10^9$	+	U	U
	4·5	2·0	$3·0 \times 10^5$	$6·0 \times 10^5$	+	U	U
NCIB	6·9	0	—	$2·7 \times 10^6$	n.a.	0·70	U
	6·9	0	$1·8 \times 10^4$	$2·7 \times 10^6$	−	0·60	U
	5·5	1·0	$1·8 \times 10^4$	$2·7 \times 10^6$	+	0·04	U
	4·5	2·0	$1·0 \times 10^4$	$1·1 \times 10^7$	+	U	U
LMD	6·9	0	—	$2·9 \times 10^6$	n.a.	2·60	0·02
	6·9	0	$1·8 \times 10^4$	$2·9 \times 10^6$	−	1·80	0·02
	5·5	1·0	$1·8 \times 10^4$	$2·9 \times 10^6$	−	1·00	0·09
	4·5	2·0	$1·0 \times 10^4$	$2·9 \times 10^6$	+	U	U

n.a., not applicable; +, good mycelial growth; U, undetectable; n.d., not determined; −, no mycelial growth, coconut medium yellow.

in agreement with the reports of Ko (1985) and Lijmbach (1969). BA levels increased significantly between 24 and 48 h incubation. The LMD strain produced considerably more BA than did the other two strains of *Ps. cocovenenans*. TF was produced at levels of less than 0·07 mg/g CCM except by strain NCIB which failed to produce it in up to 96 h incubation. Only the LMD strain produced TF after 24 h incubation.

Effect of pH

Table 2 shows that BA and TF production in CCM contaminated by *Ps. cocovenenans* strain ITB was higher at neutral than at lower pH. At pH 5·5 and 5·0 the toxins were still produced although at lower levels, especially for BA. The reduction in BA level was significant when the pH was decreased to 5·0. The reduced production of BA and of TF by *Ps. cocovenenans* strain ITB was accompanied by a decrease in the intensity of yellow coloration of the CCM. The reduction in pH to 4·5 reduced the BA level to 0·05 mg/g in the presence of an initial inoculum of $1·2 \times 10^6$ bacteria/g CCM. No TF was detectable under these conditions.

Effect of sodium chloride

Higher levels of BA but not of TF were produced in CCM at pH 6·9 containing 0·6, 1·0 and 2·0% sodium chloride (Table 2) compared to CCM of lower pH (5·5–5·0). Compared with the effect of lowering the pH, sodium chloride up to 1% shows a relatively minor effect. At neutral pH, 2% sodium chloride inhibited TF but not BA production of *P. cocovenenans* strain ITB.

Effect of pH and sodium chloride

Toxin production decreased significantly in CCM containing sodium chloride combined with a lower pH (Table 2). No toxins were pro-

duced by an initial number of $1{\cdot}2 \times 10^6$ *Ps. cocovenenans* cells/g CCM in the presence of 1% sodium chloride at pH 5·5; BA but no TF was produced with a slightly higher ($4{\cdot}3 \times 10^6$/g) initial number of bacteria. Addition of 2·0% sodium chloride to CCM at either pH 5·0 or 4·5 resulted in no BA production.

GROWTH OF *Rhizopus oligosporus* AND TOXIN PRODUCTION IN TEMPE BONGKREK CONTAINING *Pseudomonas cocovenenans*

Effect of bacterial numbers inoculated

CCM of normal pH containing *Ps. cocovenenans* at numbers about equal to that of the initial number of *R. oligosporus* spores produced well-made tempe bongkrek (Table 3). Growth of fungus was not affected as long as the initial number of bacterial cells was lower than the initial number of spores. However, well-made tempe bongkrek may still contain toxin. Tempe in which the initial number of bacterial cells was greater than the number of *R. oligosporus* spores was yellow-coloured with a spoiled smell; the fungus did not grow and the coconut medium remained as loose particles without mycelial matting.

In well-made tempe bongkrek inoculated initially with a low number of bacterial cells (2·9 cells/g CCM), BA was still produced at a low level (0·07 mg/g). A significantly higher level of BA was found when a constant number of *R. oligosporus* spores ($4{\cdot}2 \times 10^4$) was inoculated together with an equal or greater number of cells of *Ps. cocovenenans*. This is in agreement with the results of Ko *et al.* (1979).

Effect of pH

Tempe bongkrek initially containing large numbers of bacteria ($2{\cdot}9 \times 10^6$ cells/g, strain LMD) had the highest level of BA (2·6 mg/g) at a normal pH of about 6·9 (Table 3). Toxin production markedly decreased as the pH was lowered (strain ITB). At pH 5·5 BA was still produced when the initial number of *R. oligosporus* spores was lower than the initial number of bacterial cells. It is also clear that *R. oligosporus* has a suppressive effect on toxin production. However, the reduction of BA level caused by growth of the *Rhizopus* was smaller compared with that resulting from lowering the pH to 5·5. Lowering the pH to 5·0 or 4·5 inhibited toxin formation.

Effect of sodium chloride

BA production in CCM containing *Ps. cocovenenans* was little affected by 0·6% sodium chloride, but the levels of BA and TF were reduced by 47% and 60%, respectively, in CCM containing 1% sodium chloride (Table 2). When initial bacterial numbers were much higher than the initial number of mould spores, however, toxin production in tempe bongkrek was not suppressed (Table 3). Mould growth was not affected when the concentration of sodium chloride was increased to 2%. *Pseudomonas cocovenenans* strain ITB produced a small amount of BA in tempe bongkrek containing 2% sodium chloride. This finding disagrees with the results of Ko *et al.* (1979) who reported that the organism did not produce any toxins that could be detected spectrophotometrically in CCM containing 2% sodium chloride, and may reflect the greater sensitivity of the HPLC method.

Effect of pH and sodium chloride

The presence of 1% sodium chloride in CCM at pH 5·5 inhibited toxin formation in tempe bongkrek by *Ps. cocovenenans* strain ITB, but not for strain LMD and only for TF formation by strain NCIB (Table 3). Mould growth was satisfactory in tempe contaminated by strains ITB and NCIB, but was inhibited in CCM containing strain LMD. For the latter strain the levels of BA and TF were still high in the presence of 1% sodium chloride at pH 5·5, although toxin formation was inhibited in the presence of 2% sodium chloride at pH 4·5 as it was also for the other two strains. It is clear from these results that satisfactory growth of the mould is not an indication of the absence of bacterial toxins if tempe bongkrek is made from raw material contaminated with a significant number of cells of *P. cocovenenans*. The reason for the increased level of TF produced by strain LMD in the presence of 1% sodium chloride at pH 5·5 is not known and requires more detailed study.

Discussion

The addition to CCM or tempe bongkrek of salt alone, or acetic acid to a pH of 5·5, was

insufficient to inhibit the production of BA, although TF production was inhibited for *Ps. cocovenenans* strain ITB (Table 3). The production of BA and TF in either CCM or tempe bongkrek was inhibited for all strains of *Ps. cocovenanans* tested when the medium contained 2% salt and the pH was adjusted to 4·5 with acetic acid. Toxin production was inhibited at pH 5·5 in the presence of 1% sodium chloride for strain ITB, but not for strain NCIB and especially not for strain LMD.

The addition of acid and/or salt reduced toxin levels significantly, depending on the strain, but since a safe level of BA and/or TF has not been reported in the literature, any levels of these toxins in tempe bongkrek should be regarded as unacceptable until detailed toxicological data are available.

Production of BA and TF in tempe bongkrek containing *Ps. cocovenenans* is markedly affected by the growth of the mould. Although toxin production by *Ps. cocovenenans* affected the growth of *R. oligosporus*, the mould also affected bacterial growth, as shown by the fact that toxin levels in CCM (Tables 1, 2) were higher than in tempe bongkrek (Table 3), possibly due to the production of antibacterial agents by the mould (Wang *et al.* 1972).

The growth of *R. oligosporus* was not affected by addition of salt and/or acetic acid to tempe bongkrek, even when the initial number of *Ps. cocovenenans* cells/g exceeded the initial number of mould spores/g (Table 3). It is clear from the results of the present study and those of Ko & Kelholt (1981) and Ko (1985) that toxin inhibition can be assured if the raw material contains up to 2% sodium chloride and is acidified with acetic acid to pH 4.5, and if the mould inoculum exceeds that of the initial number of *Ps. cocovenenans* cells by at least a factor of 10.

Tempe bongkrek or CCM containing both toxins was yellow coloured, the extent depending on the level of TF in the samples. Tempe or CCM containing only BA was not coloured, and was characterized by a putrid odour and significant moistness when the mould either was absent or did not grow satisfactorily. However, the absence of a yellow colour in a sample of tempe bongkrek in which mould growth appears normal is not a guarantee that toxin is absent (e.g. see Table 3) unless the product contains 2% sodium chloride and has been acidified to pH 4·5 with acetic acid, at least for the strains examined in this study.

Detailed studies are continuing on the affects of other materials (e.g. organic acids, traditional acidulants, spices) on the growth of and toxin production by *Ps. cocovenenans* in CCM and tempe bongkrek. The organoleptic consequences of such treatments also will be assessed.

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References

ANON. 1988 Fourteen people die due to tempe bongkrek consumption. *Kompas* 29 February.

ARBIANTO, P. 1979 Bongkrek food poisoning in Java. In *Proceedings of the 5th International Conference on Global Impacts of Applied Microbiology, Bangkok, Thailand, 21–26 November 1977* ed. Matangkasombut, P. pp. 371–374. Bangkok: Government of Thailand.

KO, S.D. 1985 Growth and toxin production of *Pseudomonas cocovenenans*, the so-called bongkrek bacteria. *ASEAN Food Journal* **2**, 78–84.

KO, S.D. & KELHOLT, A.J. 1981 The inhibitive effect of *Rhizopus oligosporus* on toxin production by *Pseudomonas cocovenenans* in tempeh bongkrek. *Annales Bogoriensis* **3**, 97–106.

KO, S.D., KELHOLT, A.J. & KAMPELMACHER, E.H. 1979 Inhibition of toxin production in tempeh bongkrek. In *Proceedings of the 5th International Conference on Global Impacts of Applied Microbiology, Bangkok, Thailand, 21–26 November 1977* ed. Matangkasombut, P. pp. 375–378. Bangkok: Government of Thailand.

LIJMBACH, G.W.M. 1969 Bongkrekzuur, een toxine van *Pseudomonas cocovenenans*. PhD thesis, Technische Hogeschool, Delft, The Netherlands.

VAN DAMME, P.A., JOHANNES, A.G., COX, H.C. & BERENDS, W. 1960 On toxoflavin, the yellow poison of *Pseudomonas cocovenenans*. *Requeil* **79**, 255–267.

VAN VEEN, A.A. 1966 The bongkrek toxins. In *Biochemistry of Some Foodborne Microbial Toxins* eds. Mateles, R.I. & Wogan, G.N. pp. 43–50. Boston, MA: MIT Press.

VORAGEN, A.G.J., DE KOK, H.A.M., KELHOLT, A.J., SCHOLS, H.A. & KO, S.D. 1982 Determination of bongkrek acid and toxoflavin by high pressure liquid chromatography. *Food Chemistry* **9**, 167–174.

WANG, H.L., ELLIS, J.J. & HESSELTINE, C.W. 1972 Antibacterial activity produced by mould commonly used in oriental food fermentations. *Mycologia* **64**, 218–221.

WELLING, W., COHEN, J.A. & BERENDS, W. 1960 Disturbance of oxidative phosphorylation by an antibioticum produced by *Pseudomonas cocovenenans*. *Biochemical Pharmacology* **3**, 122–135.