Acta Alimentaria, Vol. 43 (3), pp. 373–377 (2014)
DOI: 10.1556/AAlim.43.2014.3.2

APPLICATION OF SYNTHETIC SOLID CULTURE MEDIUM TO IMPROVE THE DETECTION OF ANTIMICROBIAL DRUG RESIDUES IN FOODSTUFFS

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(Received: 5 November 2012; accepted: 19 March 2013)

A selective synthetic solid minimal medium (BS agar) was developed to detect antimicrobial drug-residues in foodstuffs using *Bacillus subtilis* indicator culture. This medium contains an ammonium salt as nitrogen source and either glucose or sodium pyruvate as carbon sources.

Its selectivity is based on the fact that *Bacillus subtilis* is still able to grow if the minimal medium consists of simple inorganic substances as nitrogen sources, and glucose or pyruvate as carbon supply. Using these new synthetic media for microbiological assays assessing certain antimicrobials, the diameter of the inhibition zones were 1.4–4 times wider than on the Mueller-Hinton agar.

The advantages of the BS agars are their standard compositions, the absence of inhibitors, the reproducible quality and the low costs.

Keywords: *Bacillus subtilis*, novel synthetic medium, antimicrobials, antibiotics, foodstuff, residues

The antibiotic and/or xenobiotic content of foodstuffs can be harmful for food consumers, causing allergic reactions and increased frequency of bacterial resistance to certain antimicrobial compounds used for therapeutical purposes. Microbiological techniques, ELISA and thin layer chromatography are the basic screening methods for traces of veterinary drug residues possessing antibiotic or antibacterial activity in foods of animal origin. (Bogaerts & Wolf, 1980). These microbiological methods are based on the measurement and evaluation of zones of inhibited bacterial growth on solid media (Kirbis, 2006). Under standardized conditions, the size of the inhibition is linearly proportional with the log of the drug concentration (Schoevers et al., 1994).

These assays are cost-effective and widely applied since they have the potential to cover almost the entire antibiotic spectrum within one test in contrast to the immunological or receptor based tests (e.g. apramycin cannot be detected) (Pikkemaat, 2009). The Four Plate Test (EU4pt) is a commonly used and highly appreciated method in routine work (Hussein, 2004; Kilinc & Cakli, 2008). The biggest advantage of this method is its rapidity and simplicity. At the same time, it is robust and suitable for large sample throughput, either for frozen-thawed or fresh tissues (Currie et al., 1998). Nevertheless, these methods are less specific and sensitive compared to the expensive and time-consuming chemical analysis.

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The detection of antimicrobial residues in food requires screening methods that are sensitive enough to detect antibiotic concentrations close to the maximum residue limit (MRL, EU Comission regulation (EU, 2010)). However, the establishment of MRLs has made the researchers reconsider the original microbial screening methods, such as EU4pt and one-plate *Bacillus subtilis* assays, applied in the pre-MRL era, as it was concluded that for many residues these tests are insufficiently sensitive (Currie et al., 1998; Okerman et al., 1998). In the last decade the development of improved methods accelerated. Pikkemaat (2009) and Ferrini and co-workers (2006) presented a six-plate method, the combined plate microbial assay (CPMA), which essentially consists of the EU4pt extended with additional *Bacillus cereus* and *Escherichia coli* plates.

In the six-plate standard agar diffusion tests, *B. subtilis* is used as test organism in three of the six sub-tests. Therefore, we decided to develop a new test medium, which enables a better diffusion of the antimicrobials. Synthetic media developed in our laboratory for culturing indicator microbes served as basis, as it was observed that these culture media enabled a quite intensive diffusion of bacterial metabolic products (e.g. organic acids). We assumed that the antimicrobial food residues would behave the same way, which means a more pronounced and intensive diffusion than in conventional agar plates.

There is no data available in the literature on compounds utilisable by *B. subtilis* as exclusive nitrogen sources. Considering this, first we assessed different organic and inorganic N sources. We found ammonium as highly applicable for this purpose. This is the simplest molecule for culturing Gram-negatives. Completed with glucose and pyruvate as carbon sources in phosphate buffer, it could serve as the nitrogen basis of the medium. Four novel synthetic media inoculated with *B. subtilis* were compared to the standard Mueller-Hinton agar in disc diffusion tests.

1. Materials and methods

1.1. Composition and preparation of media

Mueller-Hinton agar: Meat infusion (2.0 g l^{-1}); casein hydrolysate (17.5 g l^{-1}); starch (1.5 g l^{-1}); agar-agar (16.0 g l^{-1}). Heat treatment was carried out at 121 °C for 20 min.

BSg agar: ammonium sulphate (1 g l^{-1}), glucose (4 g l^{-1}), di-potassium hydrogen phosphate (4 g l^{-1}), potassium di-hydrogen phosphate (1 g l^{-1}), agar-agar (16.0 g l^{-1}). Heat treatment was carried out at 121 °C for 20 min.

BSp agar: ammonium sulphate (1 g l^{-1}), sodium-pyruvate (4 g l^{-1}), di-potassium hydrogen phosphate (4 g l^{-1}) potassium di-hydrogen phosphate (1 g l^{-1}), agar-agar (16.0 g l^{-1}). Heat treatment was carried out at 121 °C for 20 min.

1.2. Bacterial strain and its preparation from spore suspension

Bacillus subtilis ATCC 6633 (BGA) strain was spread onto nutrient agar pH 7.0 and, after incubation at 26 °C for 10 days, the colonies, which consisted mainly of spores, were rinsed with physiological saline solution (0.85% saline). This suspension was centrifuged at 3024 *g* for 10 minutes, washed 3 times, then the sediment was re-suspended with physiological saline solution and treated with heat at 70 ºC for 30 min. The final spore suspension was diluted to 10^7 spores per ml. The MPN-method was applied to determine the spore concentration. The suspension was stored at a temperature not exceeding 4 °C.

To obtain a 10^4 spore per ml final concentration, 0.1 ml *B. subtilis* BGA spore suspension was added to 100 ml test medium of pH 6.0 and 8.0. The agar diffusion tests for the comparison of media were performed with paper disc diffusion method, which is equally sensitive as the well diffusion method.

1.3. Resistest antimicrobial discs

For the experiments discs impregnated with standard solutions of antimicrobial drugs (Resistest HUMAN Co.) were used. Five parallel measurements were performed for each antimicrobial.

2. Results and discussion

As many as 21 antimicrobial drugs were examined during the experiments. The diameter of the inhibition zone for respective antimicrobial drugs on the Mueller-Hinton (MH) medium served as the basis of the comparison (100%). Out of the four tested aminoglycosides, the inhibition zone of neomycin was 144–164% and of gentamycin 167–200% on the new media (Table 1). Using BSg pH 6 media, kanamycin inhibited the growth of *B. subtilis* with the same diameter as on MH media. The correlated values of the diameters were wider, 191–211% on BSg pH 8 medium and BSp pH 6 and pH 8 media. The inhibitory zone of streptomycin on BSg pH 8 and BSp pH 8 was not wider than on MH medium. However, larger inhibitory zones were developed (163%) on BSg pH 6 and BSp pH 6 media. The components of the examined media significantly influenced the diameter of the inhibitory zone in case of aminoglycoside drugs.

Table 1. Microbiological assay of aminoglycosides. Size of the inhibitory zones on the medium containing *B. subtilis* after 24 h incubation (mm)

Aminoglycosides	pH 6.0					pH 8.0				
	MH	BSg	%1	BSp	%2	MH	BSg	%1	BSp	%2
Kanamycin	*9*	9	*100*	19	*211*	*11*	21	*191*	22	*200*
Neomycin	*9*	13	*144*	14	*156*	*11*	18	*164*	18	*164*
Streptomycin	*8*	13	*163*	13	*163*	*11*	11	*100*	10	*91*
Gentamycin	*7*	14	*200*	14	*200*	*9*	15	*167*	15	*167*

MH: Mueller-Hinton agar; %1: percentage of the size of the inhibitory zone on BSg agar compared to the size of the inhibitory zone on the Mueller-Hinton agar; %2: percentage of the size of the inhibitory zone on BSp agar compared to the size of the inhibitory zone on the Mueller-Hinton agar

The examined 5 penicillin derivates (methicillin, oxacillin, ampicillin, penicillin G, and cloxacillin) inhibited the growth of *B. subtilis* with wider diameters (129–260%) on all new media compared to the MH medium (Table 2). Similar results were obtained with the other 12 antimicrobial drugs, with values between 118 and 400%.

Table 2. Microbiological assay of antimicrobials other than aminoglycosides

Antimicrobials	pH 6.0					pH 8.0				
	MH	BSg	%1	BSp	%2	MH	BSg	%1	BSp	%2
Methicillin	*4*	8	*200*	6	*150*	*4*	10	*250*	8	*200*
Oxacillin	*11*	16	*146*	16	*145*	*8*	16	*200*	16	*200*
Ampicillin	*17*	22	*129*	24	*141*	*16*	22	*138*	25	*156*
Penicillin	*10*	18	*180*	22	*220*	*11*	15	*136*	22	*200*
Cloxacillin	10	25	250	26	260	10	20	200	20	200
Erithromycin	*3*	10	*333*	12	*400*	*8*	14	*175*	14	*175*
Chlortetracyclin	*8*	26	*325*	31	*388*	*8*	16	*200*	18	*225*
Chloramphenicol	*11*	19	*173*	23	*209*	*13*	19	*146*	18	*138*
Polymixin B	*3*	6	*200*	6	*200*	*4*	7	*175*	7	*175*
Cephalexin	*11*	13	*118*	23	*209*	*9*	20	*222*	13	*144*
Lincomycin	*5*	7	*140*	7	*140*	*6*	7	*117*	9	*150*
Nitrofurantoin	*13*	21	*162*	29	*223*	*12*	19	*158*	21	*175*
Sulfadimidine	*6*	19	*317*	19	*317*	*8*	25	*313*	25	*313*
Cephoperazon	*12*	17	*142*	20	*167*	*9*	17	*189*	18	*200*
Tetracyclin	*10*	21	*210*	21	*210*	*10*	19	*190*	20	*200*
Nalidix acid	*14*	21	*150*	23	*164*	*10*	20	*200*	20	*200*
Ampicillin and oxacillin	*11*	18	*164*	20	*182*	*10*	15	*150*	16	*160*

MH: Mueller-Hinton agar; %1: percentage of the size of the inhibitory zone on BSg agar compared to the size of the inhibitory zone on the Mueller-Hinton agar; %2: percentage of the size of the inhibitory zone on BSp agar compared to the size of the inhibitory zone on the Mueller-Hinton agar

In case of bacterial contamination, the interfering plaques were detectable only on the nutrient agar and not on the more selective synthetic media.

3. Conclusions

Microbiological plate methods for antimicrobial residue detection are based on the measurement and evaluation of inhibited bacterial growth zones in the media. In order to improve the efficiency of these methods, different culture media were developed. Four synthetic media containing an ammonium salt as nitrogen source, glucose or sodium pyruvate as carbon source, and buffers were investigated. The experimental media enabled more pronounced diffusion of the antimicrobials, for that reason the zones of inhibition were significantly larger.

From the examined synthetic media, the BSp pH 6 is the most recommended for the detection of antimicrobial drugs. Using this medium, all 21 examined antimicrobial drugs gave significantly larger inhibition zones with *B. subtilis* than in the conventional MH medium. The correlated values of the inhibitory zone in case of BSp pH 6 were 140–400%. In conclusion, this medium has been proven to be more sensitive in the antibiotic disc diffusion tests than the MH medium. BSp pH 6 medium will presumably behave the same way when routine samples with residues are tested. A further advantage of the novel medium is that the contaminating bacteria can impair the tests to a lesser extent than in the case of the Mueller-Hinton medium due to its highly selective nature.

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This work was supported by TÁMOP-4.2.1.B-11/2/KMR-2011-0003 research project.

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