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Research Article

A New Medium from Ram Horn Hydrolysate for Enumeration of Aerobic Bacteria

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Abstract: A new medium prepared from ram horn hydrolysate was utilized for the enumeration of aerobic bacteria from environmental samples. First, horns were ground and 35 g of horn flour was hydrolyzed chemically. The hydrolyzed material was completed to 400 ml with deionized water and this resulting solution was termed ram horn hydrolysate (RHH). It was found that it has both organic and inorganic materials sufficient for use as a bacterial medium. The RHH was enriched by the addition of glucose, yeast extract and KH_2PO_4. The effects of different concentrations (1 to 10% v/v) of RHH on the growth of bacteria from environmental samples were investigated; 4% of RHH was found to be optimal and it was called Kurbanoğlu-Algur Agar (KAA). The new medium (KAA) contains 10 g of glucose, 1 g of yeast extract, 40 ml of the hydrolysate, 1 g of KH_2PO_4 and 15 g of agar per liter of laboratory quality water. KAA medium was tested against plate count agar (PCA), nutrient agar (NA) and trypticase soy agar (TSA) for its ability to support the growth of bacteria in pure cultures and environmental samples such as soil, water, milk and meat. KAA medium yielded significantly ($P < 0.05$) higher bacterial counts than PCA and NA did, but lower (not statistically significant) counts than those of TSA. In conclusion, KAA was found to be a suitable medium for the enumeration of aerobic bacteria from environmental samples.

Key Words: Bacterial medium, fibrous proteins, horn, peptone, protein hydrolysate.

Aerobik Bakterilerin Sayımı İçin Koç Boynuzu Hidrolizatından Yeni Bir Besiyeri

Özet: Koç boynuzu hidrolizatından hazırlanan yeni bir besiyeri çevresel numunelerdeki aerobik bakterilerin sayımı için kullanıldı. Önce boynuzlar öğütüldü ve 35 g boynuz unu kimyasal olarak hidroliz edildi. Hidrolize materyal deiyonize su ile 400 ml'ye tamamlandı ve bu son solüsyon Koç Boynuz Hidrolizatı (KBH) olarak isimlendirildi. Bir bakteriyal besiyeri olarak kullanılmasına yetecek kadar organik ve inorganik maddelere sahip olduğu bulundu. KBH glikoz, maya ekstraktı ve KH_2PO_4 ilave edilerek zenginleştirildi. Farklı konsantrasyonlardaki (% 1-9 ve 10 v/v) KBH'nin çevresel numunelerdeki bakterilerin üremesi üzerindeki etkileri incelendi ve % 4'lük KBH'nin optimal olduğu bulundu ve % 4'lük KBH Kurbanoğlu-Algur Agar (KAA) olarak isimlendirildi. Yeni besiyeri (KAA) laboratuar kalitesindeki bir litre su içerisinde 10 g glikoz, 1 g maya ekstraktı, 40 ml hidrolizat, 1 g KH_2PO_4 ve 15 g agardan hazırlandı. KAA besiyeri, saf kültürler ve toprak, su, süt ve et gibi doğal örneklerdeki bakterileri üretme potansiyellerini belirlemek için, plate count agar (PCA), nutrient agar (NA) ve trypticase soy agara (TSA) karşı test edildi. Dökme plak çalışmalarından elde edilen sonuçlar, KAA besiyerinin PCA ve NA besiyerlerine göre önemli derecede ($P < 0,05$) yüksek bakteri sayısı oluşturduğunu gösterdi, fakat bu değerler TSA'dan elde edilen değerlerden biraz daha düşüktü (istatistiki bakımdan önemsiz). Sonuç olarak, KAA'nın çevresel numunelerdeki aerobik bakterilerin sayımı için besiyeri olarak uygun olduğu bulundu.

Anahtar Sözcükler: Bakteriyal besiyeri, fibröz proteinler, boynuz, pepton, protein hidrolizatı.

Introduction

There have been many papers comparing various media for their ability to support the growth of the largest number of bacteria from environmental samples (1-3). In addition to providing the highest bacterial counts, medium should also be clear or only slightly colored, prevent growth of large spreading colonies, and be free of precipitates so that small colonies may be seen.

One of the most widely accepted mediums for viable counts of bacteria from environmental samples is trypticase soy agar (TSA) (2-4). On the other hand, in the United States the method generally used to determine the bacterial count of water or wastewater is the pour plate procedure, using plate count agar (PCA) and incubation for 48 h at 35 °C (5). In addition, nutrient agar (NA) is a basic culture medium used to subculture and enumerate viable bacteria (6).

Ram horns, which are discharged at the rate of about 600 t a year by slaughterhouses in Turkey, are significant waste products of the meat industry. Fibrous proteins such as horn, feather, nail and hair are also abundant waste products. These waste products can be converted to biomass, protein concentrate or amino acids using proteases originated from certain microorganisms (7). Horns consist of α-keratin, which has a very high cysteine content (up to 22%). They also contain most of the common amino acids and have the components of bone and blood tissues. Horns are rich in some growth factors required by microorganisms (7-10). Ram horn protein hydrolysate has been investigated only to a minor extent, and its use in industrial processes is still poor (11).

Peptones are defined as protein hydrolysates that are soluble in water and not heat coagulable (12). These products may have major importance for the fishing industry as their market prices are somewhat higher than common by-products such as fish silage and fish meal. Growth substrates make up the major part of the production cost of microbial cells and by-products from the fermentation industry (13). The nitrogen source is usually the most expensive component within bacterial growth substrates, and at present it is obtained from plants (14), dairy proteins such as casein (15) or whey (16), and slaughterhouse waste. On the other hand, peptones and fish hydrolysates are made either by acid hydrolysis or the enzymatic digestion of proteins. Acid hydrolysis allows high yields; however, this process results in high ash content in the final products as the neutralization step cannot be avoided (17).

As we have performed many studies on ram horn peptone in our laboratory, we planned to compare a culture medium from ram horn peptone with standard media. Some previous experiments in our laboratory have been performed on ram horn protein hydrolysate (7,11,18-22), but this is the first time that a cultural medium prepared from a ram horn peptone has been tested for the enumeration of aerobic bacteria.

It was assumed that horn material could be used as a raw material for bacteriological media. For this reason, we investigated the suitability of horn hydrolysate as an enumeration medium for aerobic bacteria from environmental samples.

Materials and Methods

Materials

Horns were collected from Erzurum Slaughterhouse in Turkey. Bacteria: *Bacillus cereus* NRRL-3711, *Bacillus subtilis* NRS-744, and *Lactobacillus bulgaricus* B-548 were obtained from Dr C.P. Kurtzman (National Center for Agricultural Utilization Research, USA). *Streptococcus thermophilus* 70885 MCG-50 and Salmonella sp. were obtained from Dr. M. Kaya (Food Engineering Department, Agricultural Faculty, Atatürk University, Erzurum, Turkey). *Escherichia coli, Enterobacter aerogenes, Staphylococcus aureus,* Citrobacter sp., Corynebacterium sp. and Proteus sp. were isolated from patients by Dr. A. Kadanalı (Yakutiye Hospital in Erzurum, Turkey). *Pseudomonas putida* 39/D was obtained from Dr. Diana Cruden (Iowa University, USA). *Listeria monocytogenes* B_2 was isolated by us from white cheese. All strains were maintained between passages on nutrient agar slants at 5 °C.

Methods

Hydrolyzation procedures of horn: Horns were washed with deionized water and dried in an oven at 100 °C. The dry horns were cut into smaller pieces and ground with a grinder (Wiley-Mill, Arthur, USA). This ground material was termed horn powder (HP). Thirty-five grams of the HP was mixed with 50 ml of 6 N HCl. The mixture was incubated at 80 °C for 24 h. At the end of first incubation period, to the mixture was added 100 ml of deionized water and again incubated at 130 °C for 1 h. The solution was then cooled and its pH was adjusted to 7 with 10 N NaOH. It was filtered twice through Whatman No. 1 filter paper. The volume was completed to 400 ml with deionized water. The final clear filtrate was named ram horn hydrolysate (RHH) and stored at 4 °C. As a result of these procedures, 30 g of 35 g HP was hydrolyzed (7,18,19). Various concentrations (1-9 and 10%) of the RHH were enriched with 1% glucose (Oxoid), 0.1% yeast extract (Oxoid) and 0.1% KH_2PO_4. 3 H_2O (Difco). These diluted solutions were supplemented with 1.5% w/v of agar. The pHs of the media were adjusted to 7.2 with 1 N HCl and they were autoclaved at 121 °C for 15 min and tested for enumeration of bacteria from environmental samples. Four percent RHH was found to be optimal and this 4% RHH was called Kurbanoğlu-Algur Agar (KAA).

Analysis of RHH: Amino acid analysis of RHH was carried out after hydrolysis with 6 N HCl at 110 °C for 24 h in a Biotronic LC-5001 Amino Acid Analyzer (Germany). Total sugar content, dry matter and ash analysis were estimated according to Association of Official Agricultural Chemists' (AOAC) methods (23). Total nitrogen was estimated by the micro-Kjeldahl method (24). Total lipids were determined according to Folch et al. (25). The elemental composition was measured by atomic absorption spectrophotometer (Perkin Elmer, UV HS-360, Germany).

Plating method and sample collection: Bacterial colony counts were determined by the pour plate technique (5).

Three different water samples [a spring water sample, a river water (Aras River) sample and a tap water sample] were used in this study. Water samples were collected in sterile 1-l polypropylene wide-mouth bottles containing 1.0 ml of a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) to neutralize residual chlorine in the sample (5).

Three different soil samples (a forest soil sample, a greenhouse soil sample and a garden soil sample) were used in this study. The collection, preparation and inoculation procedures of the soils were carried out according to the AOAC (23).

Food samples (a raw milk sample, a pasteurized milk sample and a fresh milk sample; meat: from a market, from a butcher, from a slaughterhouse) were purchased from local retail markets and analyzed in triplicate. Food samples were collected and prepared as described in the compendium of methods for the microbiological examination of foods (26). All samples were returned to the laboratory and processed within 2 h of collection. Inoculated plates were incubated at 35 °C and colony counts were performed at 24, 48 and 72 h.

Colony counts for pure cultures: Pure cultures were maintained on slopes of nutrient agar (Oxoid) by storage at 5 °C after overnight growth at 35 °C. Broth cultures were obtained by growth at 35 °C for 24 h in nutrient broth of 100 ml in 250 ml conical flasks shaken continuously in an orbital shaker (ROSI 1000 Thermolyne). Triplicate plates were prepared for each culture and inoculated plates were incubated at 35 °C. Colony counts on NA, PCA, TSA and KAA were made at 48 h (27,28).

Statistical analysis

All media were compared against each other. The experiments were replicated 3 times in a randomized block design. All data were analyzed using the general linear models procedure of SAS. Differences among means were tested for significance ($P < 0.05$) by Duncan's multiple range tests.

Results

The main chemical composition of RHH is shown in Table 1. These data show that RHH was rich in both organic and inorganic materials. Notably, it contains the essential substances required in a bacteriological media such as sources of carbon, nitrogen and minerals. In addition, RHH is rich in amino acid content. The essential amino acids are included and among them arginine (4.66 mg ml^{-1}) is the highest. However, of all the amino acids considered, glutamic acid (8.17 mg ml^{-1}) was the most abundant. Obtained cysteine content was lower than that of some other fibrous proteins.

First, we investigated the effects of RHH in various concentrations (1-9 and 10%) on the colony counts. We found that the most appropriate concentration of RHH for growth was 4% and the colony yields (log CFU ml^{-1}) for this application were 7.22 of soil, 4.38 of water, 7.44 of meat and 6.33 of milk (Figure). The 4% RHH was called KAA. The main chemical composition of KAA (l^{-1}) is 40 ml of RHH, 15 g of agar agar, 10 g of glucose and 1 g of KH_2PO_4. A medium containing all of the chemicals except for RHH was used for enumeration, but no growth was observed.

Table 2 shows the results of aerobic bacterial counts from the samples of soil, water, milk and meat on NA, PCA, TSA and KAA. PCA plates yielded the poorest results with all the samples tested. The total bacterial counts (log CFU ml^{-1}) obtained from TSA, KAA, NA and PCA were 264.84, 259.54, 229.57 and 226.11, respectively.

We continued the tests on pure cultures belonging to different physiological groups of bacteria, and the results are given in Table 3. In accordance with the results in Table 2, KAA and TSA yielded higher bacterial counts than NA and PCA.

Table 1. The main chemical composition of RHH.

Components g ml^{-100}		Amino acids mg ml^{-1}	
Nitrogen	0.881	Aspartic acid	3.90
Protein	5.500	Threonine	2.00
Dry matter	12.80	Serine	2.87
Ash	4.98	Glutamic acid	8.17
Total sugar	0.500	Glycine	5.19
Total lipids	0.300	Alanine	3.19
Mg	0.160	Cysteine	0.21
Ca	0.164	Valine	2.56
Cu	0.017	Methionine	0.41
Mn	0.036	Isoleucine	1.63
Zn	0.064	Leucine	4.02
Fe	0.123	Tyrosine	1.61
K	0.113	Phenylalanine	1.67
		Histidine	0.72
		Lysine	2.21
		Arginine	4.66
		Proline*	3.90

*Proline in RHH was determined according to Bathes et al. (29).

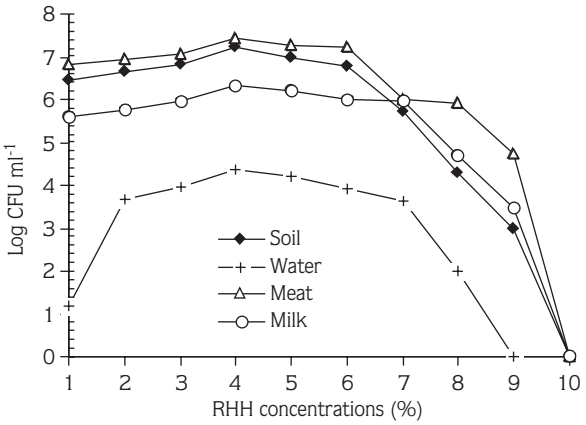

Figure. The effects of different RHH concentrations on the number of bacteria from natural samples.

Discussion

The results in Table 1 were discussed in our previous studies (7,11,18,19). It was found that RHH applications higher than 4% had an inhibiting effect. For example, the lowest colony yields (3 of soil, 0.0 of water, 4.72 of meat and 3.47 of milk) were obtained from the application of 9% RHH. Furthermore, no growth was observed from the application of 10% of RHH (Figure). This inhibiting effect may be due to the high biochemical oxygen demand (BOD) load of RHH and the presence of cell wall cations and some toxic materials. Similar effects have been observed from effluents with high loads of organic and inorganic materials (30). Therefore, we continued researching (comparison with other media) with 4% RHH.

The differences between the results of NA and PCA are not significant ($P < 0.05$). Similarly, the differences between the results of TSA and KAA are also not significant. However, the differences between the results of TSA-KAA and NA-PCA were found to be significant ($P < 0.05$). On the other hand, after 24 h of incubation for water (sample no. 1 and 3), no growth was observed on NA and PCA. For the same samples, the mean colony counts (log CFU ml^{-1}) on TSA and KAA were 2.64 and 2.04 (sample no. 1 and 3) and 2.26 and 1.94 (sample no. 1 and 3), respectively. There was also no significant difference between KAA and TSA for these samples. As seen in Table 2, the third water sample is tap water that is chlorinated water and may contain injured bacteria that were unable to grow in PCA and NA. Rodrigues and Kroll (27) reported that many types of physical or chemical stresses have been used to eliminate or control bacteriological growth in products. This application results in the sublethal injury of cells unable to grow under certain environmental contitions and renders the cells susceptible to secondary stresses such as the selective agents commonly used in media for their enumeration. These organisms can pass undetected during routine bacteriological examinations but, because they are capable of recovery and growth following the

Table 2. Comparison of growth of total aerobic bacteria in natural samples on NA, PCA, TSA and KAA.

Samples	Incubation time (h)	Sample No.	Log CFU ml^{-1} on NA	PCA	TSA	KAA
		1	9.54 a	9.44 a	10.72 b	10.60 b
	24	2	4.80 a	4.70 a	4.90 b	4.85 b
		3	9.80 a	9.70 a	10.86 b	10.40 b
		1	9.90 a	9.94 a	11.14 b	10.97 b
Soil*	48	2	5.00 a	4.95 a	5.48 a	5.44 a
		3	12.01 a	11.95 a	13.01 b	12.98 b
		1	10.14 a	10.06 a	12.19 b	12.11 b
	72	2	5.72 a	5.54 a	6.86 b	6.68 b
		3	11.19 a	11.10 a	12.19 b	12.16 b
		1	NG a	NG a	2.64 b	2.26 b
	24	2	3.16 a	3.15 a	3.48 a	3.79 a
		3	NG a	NG a	2.04 b	1.94 b
		1	2.90 a	2.75 a	3.65 b	3.52 b
Water**	48	2	3.66 a	3.80 a	4.51 b	4.34 b
		3	NG a	NG a	2.45 b	2.20 b
		1	2.63 a	2.54 a	3.56 b	3.40 b
	72	2	4.03 a	3.98 a	4.98 b	4.90 b
		3	2.24 a	2.18 a	3.19 b	3.15 b
		1	4.90 a	4.80 a	5.76 b	5.49 b
	24	2	9.79 a	9.57 a	10.24 b	10.18 b
		3	9.60 a	9.66 a	10.48 b	10.27 b
		1	5.90 a	5.80 a	6.70 b	6.60 b
Meat***	48	2	10.56 a	10.24 a	11.74 b	11.58 b
		3	10.65 a	10.60 a	11.28 b	11.18 b
		1	6.18 a	6.14 a	6.98 b	6.76 b
	72	2	11.13 a	11.10 a	12.88 b	12.13 b
		3	11.17 a	11.11 a	12.14 b	11.98 b
		1	6.90 a	6.53 a	6.98 a	6.85 a
	24	2	3.70 a	3.18 a	4.18 b	4.14 b
		3	4.10 a	4.14 a	4.98 b	4.82 b
		1	7.94 a	7.84 a	8.35 b	8.19 b
Milk****	48	2	4.85 a	4.62 a	5.82 b	5.73 b
		3	5.88 a	5.66 a	6.44 b	6.24 b
		1	8.27 a	8.26 a	8.94 b	8.84 b
	72	2	5.10 a	4.98 a	6.11 b	5.99 b
		3	6.23 a	6.10 a	6.99 b	6.88 b
Total colony counts			229.57 a	226.11 a	264.84 b	259.54 b

[a,b] Means in row without a common superscript differ ($P < 0.05$). Means of 3 trials, and each trial were examined in duplicate. Values with the same letter are not significant ($P < 0.05$).

* Sample 1:Greenhouse soil; 2: Garden soil; 3: Forest soil.

** Sample 1: Spring water; 2: Water of Aras River; 3: Tap water.

*** Sample 1: From a market; 2: From a butcher; 3: From a slaughterhouse.

**** Sample 1: Untreated milk; 2: Pasteurized milk; 3: Fresh milk. NG: No growth

Table 3. Comparison of growth of test bacteria on NA, PCA, TSA and KAA.

Bacteria	Log CFU ml^{-1} on			
	NA	PCA	TSA	KAA
Bacillus cereus NRRL-3711	7.04 a	5.90 b	8.06 c	8.00 c
Bacillus subtilis NRS-744	11.13 a	11.13 a	11.10 a	11.12 a
Lactobacillus bulgaricus B-548	8.60 a	8.01 b	8.80 c	8.75 c
Eschherichia coli	9.01 a	9.00 a	9.03 a	9.10 a
Listeria monocytogenes B_2	12.16 a	12.04 a	12.13 a	12.14 a
Streptococcus thermophilus 70885 MCG-50	8.44 a	8.06 a	9.12 b	9.10 b
Enterobacter aerogenes	11.01 a	11.10 b	11.10 b	11.12 b
Pseudomonas putida 39/D	11.41 a	11.31 a	12.11 b	12.03 b
Staphylococcus aureus	11.21 a	11.18 a	11.25 a	11.20 a
Citrobacter sp.	11.28 a	11.34 a	11.37 a	11.35 a
Proteus sp.	HG	HG	HG	HG
Corynebacterium sp.	10.17 a	10.13 a	10.68 b	10.72 b
Salmonella sp.	11.07 a	11.00 a	11.04 a	11.03 a
Total colony counts	122.53 a	120.2 a	125.79 b	125.66 b

Values with the same letter are not significant ($P < 0.05$). HG: Heavy growth (it could not be counted)

removal of the stress, they can represent a considerable health hazard. Hurst (31) reports that a medium quite suitable for an uninjured organism may become inadequate for an injured one. It is thus essential to use enumeration media that allow the growth of both injured and noninjured bacteria. TSA is recommended for the growth and enumeration of injured bacteria in environmental samples (2,32,33). For this reason, we selected TSA for comparison with KAA. The results in Table 2 show that TSA and KAA are better than NA and PCA in supporting higher numbers of colonies for both injured and noninjured bacteria.

As seen in Table 3 the differences between the results obtained from 6 of the test organisms (*B. subtilis, E. coli, L. monocytogenes, S. aureus,* Citrobacter sp. and Salmonella sp.) are not significant ($P < 0.05$) for all of the media, while TSA and KAA were statistically better than PCA and NA for other 6 test organisms. This shows that KAA does not demonstrate inhibitory effects on the organisms tested to any greater extent than standard media. On the other hand, Kurbanoglu and Algur (18) reported that when spraying 5000 ml of 2% RHH onto each tray (0.94 m^2), increases in *Agaricus bisporus* yield of 38% were obtained. According to this report, RHH need to be thoroughly investigated to find industrial applications.

It was reported that a microbiological medium should be clear or only slightly colored, prevent growth of large spreading colonies, and be free of precipitates so that small colonies may be seen (4). KAA has the properties mentioned above. Based on our results we think that KAA can be used as a medium for the enumeration of aerobic bacteria from environmental samples or at least for general microbiological purposes. On the other hand, one source (34) reported an acid hydrolysis process for whole fish, where steam distillation was used to remove aromatic substances followed by filtration and then concentration. The concentrate was used in dehydrated soup cubes and as a microbial media. Acid hydrolysis is also widely utilized to convert underutilized and secondary raw material from fish into fertilizer due to the low production cost and resulting extensive hydrolysis. In this study, RHH was considered as a supplement in the fermentation medium because it contained various amino acids and minerals.

Consequently, we prepared 10 l of bacterial medium from 35 g of HP and converted it into a useful product. Waste horns can cause severe environmental problems due to the associated high organic pollutant (BOD) and microbial loads. They are disposed of by municipal councils in Turkey. Bacterial-medium recovery from waste horns in Turkey could somewhat reduce this

pollution problem. Equally, this process may be a very economical method, especially for countries that need to import a source of bacterial medium. Other fibrous proteins such as feather, nail and hair should be considered for bacterial medium production as well.

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