THE USE OF WASHED AGAR IN CULTURE MEDIA

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In connection with the preparation of milk-powder agar it was found that it was necessary to wash the agar in order that it might be used in the medium without causing a precipitate during sterilization. Qualitative tests indicated that the calcium and magnesium salts in the agar probably combined with the phosphate used in the preparation of the milk-powder solution and the phosphates were precipitated during sterilization. To overcome this difficulty, the agar was washed in distilled water in order to reduce its salt content.

Soil bacteriologists have employed washed agar for many years. It has been washed in order to remove as much as possible of the soluble organic matter which is believed to be detrimental to the growth of nitrifying bacteria. Sternberg in his "Textbook of Bacteriology" recommended that agar be soaked in cold water for twenty-four hours; this was advised in order to facilitate dissolving and filtering. It is the practice in some laboratories to wash agar, apparently more for the sake of a cleaner preparation than for any other reason.

Washed agar gave such satisfactory results in the milk-powder medium that its use in the regular standard peptone extract medium was tried. It is the purpose of this paper to present briefly the results of this work and to show the effect of washing on the calcium and magnesium content of agar.

The results in table 1 show in a decided manner the effect on the bacterial count of using washed agar. While there was not an increase in all samples, there was in many cases an increase beyond any experimental error. As a rule, the higher counts were found when pasteurized milk was examined.

TABLE 1

Bacteria counts on standard extract agar using shred and washed agar

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	APPROXIMATE INCREASE OVER STANDARD EXTRACT AGAR
Raw milk		
bacteria per cc.	*bacteria per cc.*	*per cent*
650,000	990,000	52.0
1,110,000	1,530,000	37.5
1,410,000	1,940,000	37.5
1,160,000	5,180,000	345.0
1,580,000	3,620,000	129.0
246,000	432,000	75.0
239,000	1,010,000	320.0
3,370,000	11,580,000	246.0
1,760,000	3,040,000	72.6
2,300,000	4,330,000	88.2
5,120,000	15,360,000	199.0
139,000	198,000	42.5
263,000	346,000	31.5
720,000	1,010,000	40.0
990,000	800,000	−23.0
130,000	224,000	72.0
154,000	259,000	68.2
141,000	144,000	2.1
620,000	790,000	27.0
196,000	232,000	18.4
184,000	230,000	25.0
167,000	189,000	13.2
9,700,000	16,800,000	73.0
30,300,000	50,400,000	66.0
15,700,000	3,200,000	−20.0
4,200,000	3,500,000	−8.0
339,000	264,000	−7.8
428,000	189,000	−44.0
6,500,000	6,100,000	−9.5
48,500,000	36,300,000	−7.5
287,000	228,000	−8.0
1,420,000	1,050,000	−7.0
830,000	740,000	−9.0
Pasteurized milk		
1,600	2,100	31.0
1,000	8,000	700.0
3,500	17,000	385.0
41,900	63,000	50.0

TABLE 1—*Continued*

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	INCREASE OVER STANDARD EXTRACT AGAR
Pasteurized milk—*continued*		
bacteria per cc.	*bacteria per cc.*	*per cent*
13,000	22,000	69.0
4,800	66,000	1275.0
45,000	76,000	69.0
5,700	68,000	1090.0
1,500	20,100	1240.0
52,000	84,000	61.5
1,400	3,100	121.0
580,000	870,000	50.0
44,000	94,000	135.0
12,500	18,600	48.0
13,200	19,700	48.0
62,000	88,000	42.0
13,100	15,700	19.0
6,400	37,000	480.0
10,000	14,900	49.0
9,600,000	11,520,000	19.0
34,800	71,000	104.0

The washed agar used in these experiments was prepared as follows: For 1 liter of double strength (3 per cent) agar, 30 grams of shred agar was placed in a flask with 2000 cc. of distilled water. This was allowed to stand for 24 hours at room temperature. At the end of this period as much water as possible was poured off, a piece of cheesecloth being placed over the top of the flask. Fresh distilled water was added to make up for the water poured off. The agar was allowed to soak another twenty-four hours, after which it was thrown on to a cotton-flannel cloth in a funnel and washed once with 1 liter of distilled water. The agar was allowed to drain and as much as possible of the remaining water was pressed out by squeezing the filter cloth with the hands. A container large enough to hold the agar was counterpoised on the laboratory scales, and the agar was placed in it. In the opposite pan was placed 30 grams for the weight of the agar and 1000 grams for the weight of the water in which it was to be dissolved. Then water enough was added to the agar to make up this weight, it was then dissolved and filtered.

After the agar is washed, it may be air dried, and then used the same as ordinary agar. It is only necessary to use 1 per cent of the dry material to obtain a medium having the same jelly strength as a 1.5 per cent shred agar medium.

Various methods were tried for washing shred agar, and the effect of these treatments on the calcium and magnesium content will be of interest. As shown in table 2, ordinary shred agar contained about 16 per cent of moisture. On an air-dry basis

TABLE 2

Analyses of various kinds of agar

KIND OF AGAR	MOISTURE	ON DRY BASIS			
		Ash	CaO	MgO	Protein
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Shred agar					
Sample 1	16.37	4.46	1.17	0.79	2.29
Sample 2	15.97	4.48	1.13	0.76	2.23
Commercially purified agar					
Lot No. 1	12.83	4.54	1.41	0.64	1.66
Lot No. 2	8.82	3.77	1.12	0.55	1.40
Washed agar					
24 hours (A)	14.21	3.37	0.74	0.53	
48 hours (B)	14.62	2.99	0.70	0.39	
72 hours (C)	15.45	2.74	0.61	0.34	
24 hour washed 30 grams to 5000 H_2O	14.32	2.74	0.61	0.43	
NaCl-acid treated agar	12.70	3.37	0.39	0.08	1.35

the per cent of ash was about 4.4. The calcium content was about 1.15 per cent, calculated as CaO, and the magnesium about 0.77 per cent, calculated as MgO.

Two lots of commercially purified agar were examined. These were purchased at different times, and probably do not represent one orginal lot of the material. The agar was said to contain a minimum amount of moisture, when packed, and to have been specially treated to reduce the extraneous matter, inorganic

salts, and acidity. As the results show, the moisture content of this agar was variable, but the content of calcium was no lower than that of shred agar, although the magnesium had been slightly reduced. The protein content, however, was about 50 per cent of that of shred agar.

The analysis of washed agar showed a decided decrease in the percentage of calcium and magnesium, and experiments were made to determine the time required to wash the agar, starting with 30 grams to 2000 cc. of water. Agar "A" was held twenty-four hours. Agar "B" was held forty-eight hours, but after the first twenty-four hours the 2 liters of water were replaced with fresh water. Agar "C" was held seventy-two hours, the water being removed and replaced with fresh distilled water at the end of each twenty-four hours. The agar was air dried by spreading it on filter papers after washing.

It will be seen from the table that the moisture content of the three lots of agar was very similar to that of shred agar. The most interesting effect of washing was the reduction of the calcium and magnesium salts. The forty-eight-hour period of washing, which is the one we have used most extensively, reduced the CaO from about 1.1 per cent to 0.7 per cent, and the MgO from about 0.78 per cent to 0.39 per cent. The calcium and magnesium content was reduced sufficiently by this treatment to give satisfactory results in the milk-powder agar medium; that is, enough was removed so that the phosphates did not precipitate upon sterilization. Agar washed twenty-four hours in the proportion of 30 grams to 2000 cc. of water was not entirely satisfactory and, as the results show, there was but little further reduction in the calcium and magnesium by washing for seventy-two hours.

It was found that by increasing the amount of distilled water, good results could be obtained in twenty-four hours. When 30 grams of shred agar was held in 5000 cc. of distilled water for twenty-four hours the calcium and magnesium content was found to be 0.61 per cent and 0.43 per cent respectively, when calculated on a moisture-free basis.

The results obtained by the addition of NaCl and HCl to the wash water are interesting. Various amounts of salt and acid were used, their use having been suggested by Dr. Zoller of these laboratories. The best results were obtained as follows: 20 grams of shred agar was added to 1000 cc. of distilled water containing 10 grams of NaCl and 5 cc. of N/10 HCl. This was allowed to stand for six hours at room temperature, then the salt and acid solution was poured off and replaced by 1800 cc. of fresh distilled water. This was allowed to stand 18 hours, which made the washing period twenty-four hours. The agar was then poured on a cotton-flannel cloth in a funnel and washed with 500 cc. of distilled water, then allowed to drain and as much water pressed out by hand as possible. The agar was finally air dried.

The agar treated in this manner showed a lower calcium and magnesium content than any of the others. Reference to the table shows that it contained 0.39 per cent of CaO, and 0.08 per cent MgO, while the protein content was reduced from about 2.2 per cent to 1.35 per cent.

Media made with this agar did not, however, give quite such satisfactory counts as that made with washed agar, but its use has not been tried out very extensively. This method of washing, however, has possibilities of considerable value. The principal point of interest at present in connection with the use of NaCl and HCl is the fact that they assist in the removal of the calcium and magnesium salts.

We were very much interested in the results obtained with washed agar. It was valuable to us because it made possible a milk-powder agar medium which would stand sterilization without precipitation of the phosphates, but the fact that higher bacterial counts were often obtained when it replaced ordinary shred agar in the standard extract medium led us to wonder just what might be the explanation.

In connection with the milk-powder medium it had been noticed that the presence of too much phosphate often lowered the bacterial counts. Even the increase from 0.1 per cent to 0.2 per cent seemed to have a marked effect. Besides this, in

certain milk-powder media the milk powder was dissolved in such a way as to leave the calcium phosphate in the medium. With such media low counts were noticed.

Having these facts in mind and knowing that at least one of the effects of washing agar was a partial removal of the calcium and magnesium salts it occurred to us that this lowering of the percentage of these salts might be one of the reasons for the increased counts when washed agar was used in the standard medium. To confirm this opinion, counts were made on three media, all of which contained 0.5 per cent peptone and 0.3 per cent extract, and had the same reaction but made up with different kinds of agar. One contained regular shred agar, one washed agar, and the other washed agar with sufficient $CaSO_4$ and $MgSO_4$ added to make up for the calcium and magnesium (calculated as CaO and MgO) removed by washing.

TABLE 3

Effect of addition of calcium and magnesium salts to a washed-agar medium

STANDARD EXTRACT AGAR, A. P. H. A. 1916	STANDARD EXTRACT WASHED-AGAR AND Ca AND Mg SALTS	STANDARD EXTRACT WASHED AGAR
bacteria per cc.	*bacteria per cc.*	*bacteria per cc.*
6,200	5,800	8,800
13,100	8,100	15,700
6,400	6,700	37,000
10,000	12,000	14,900
9,600,000	9,550,000	11,500,000
34,800	30,600	71,000

A few samples of pasteurized milk were plated on these three media. The results in table 3 indicate that the addition of these salts to the washed-agar medium reduced the counts so that they agreed with those on the regular agar medium.

We do not intend to convey the idea that this is an explanation of the higher counts. Perhaps similar results may never be obtained again, but at least they indicate that these salts may play an important part in culture media.

SUMMARY AND CONCLUSIONS

1. The standard extract medium with washed agar showed in many cases, when market milk was examined, a much higher count than the same medium with regular shred agar.

2. Washing agar reduced its content of calcium and magnesium salts.

3. A few experiments indicated that the removal of these salts was a factor in the cause of the higher counts. This point, however, is merely suggested by the results and not definitely proved.

4. Since certain samples of milk show a higher count when plated on a washed-agar standard extract medium than on the regular standard extract medium, it seems evident that washing removes something detrimental to the growth of certain species of bacteria. This naturally suggests that a further study is needed of the value of washed agar in lines of bacteriological work where it has not been used.